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13. ABSTRACT (Maximum 200 Words) The long-term objective of this project is to develop a new treatment for breast cancer based on blockade of the autocrine growth factor activity of procathepsin D, the enzymatically inactive precursor of common enzyme cathepsin D. This precursor has been found to be overexpressed and actively secreted from various breast tumor cells. For this project it is important to realize that the aspartic proteinase cathepsin D is generated by removal of an activation peptide (APpCD) from the procathepsin D molecule. In past, procathepsin D has been identified as an independent prognostic factor in several forms of cancer, particularly breast cancer. In preliminary experiments done by our group more than 8 years ago, procathepsin D was found to act as a specific autocrine growth factor for breast cancer-derived cells, but not for any other cell type tested. Only experiments performed much later using an additional panel of cancer cell lines revealed that procathepsin D acts as a mitogen also on some prostate and ovarian cell lines.				
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INTRODUCTION

The long-term objective of this project is to develop a new treatment for breast cancer based on blockade of the autocrine growth factor activity of procathepsin D, the enzymatically inactive precursor of common enzyme cathepsin D. This precursor has been found to be overexpressed and actively secreted from various breast tumor cells.

For this project it is important to realize that the aspartic proteinase cathepsin D is generated by removal of an activation peptide (APpCD) from the procathepsin D molecule. In past, procathepsin D has been identified as an independent prognostic factor in several forms of cancer, particularly breast cancer. In preliminary experiments done by our group more than 8 years ago, procathepsin D was found to act as a specific autocrine growth factor for breast cancer-derived cells, but not for any other cell type tested. Only experiments performed much later using an additional panel of cancer cell lines revealed that procathepsin D acts as a mitogen also on some prostate and ovarian cell lines.

These growth-promoting effects were mediated through a specific receptor expressed on breast cancer cell lines that is distinct from the usually proposed cathepsin D-specific mannose-6-phosphate receptor. The region of procathepsin D responsible for its mitogenic activity was localized to APpCD and amino acids 27-44 of the APpCD sequence. No growth factor activity could be shown with the mature enzyme cathepsin D. The specific aims proposed in this application are based on the central hypothesis that procathepsin D is involved in breast cancer via a specific receptor that mediates autocrine activation for increased metastatic growth. This project proposed the following aims: 1) it is hypothesized that the overproduction of procathepsin D results in an increase in the metastatic potential of breast tumor cells. A low metastatic human breast cancer cell line will be transfected with human procathepsin D cDNA such that the cells will secrete constitutively varying amounts of procathepsin D. The metastatic potential of each transfected cell line will be evaluated both *in vitro* and *in vivo* in relationship to the amount of procathepsin D secretion. 2) Attempts will be made to determine the exact site in procathepsin D responsible for breast cancer cell growth factor activity. Synthetic peptides representing fragments of APpCD will be prepared. Amino acid substitutions in the most active peptide fragment will be used to map the essential amino acid contact sites for the receptor. 3) It is hypothesized that inhibition of the APpCD interaction with its receptor will result in inhibition of cancer cell growth. Peptide analogs will be prepared with D-amino acids to block the growth and malignancy of cancer cells.

BODY

TASK 1

MDA-MD-231 cell line were transfected with various human procathepsin D cDNA and tested for secretion of procathepsin D *in vivo*. In addition, the growth pattern *in vivo* have been established. Preliminary results shown in Figures 1 and 3 (see Appendices) showed that the cells differ in their ability to growth *in vivo* based on level of procathepsin D

secretion. These data were later confirmed using an ELISA assay employing anti-activation peptide antibodies (Figure 2) when we compared the levels of procathepsin D secreted into tissue culture supernatants of transfected and parental cells. For more details see (Vetvicka et al. 2002).

TASK 2

Subsequent experiments (Figure 4) demonstrated that mice can be protected against breast cancer growth by repeated injections with anti-procathepsin D antibodies or antibodies against syntetic fragments AA 27-44 or AA 36-44. In additional experiments, these antibodies protected mice against secondary challenge with prostate cancer cell line (Figure 5). Synthetic peptides with a single amino acid substations have been synthesized, subsequently, monoclonal antibodies against these peptides were prepared. Using this library of syntetic peptides covering the binding site AA 36-44, we establish the molecular moiety responsible for binding the activation peptide. For more details, see (Vetvicka et al. 2000, Vetvicka et al. 2002).

TASK 3

Various cytokines are involved in both cancer development and in defense against cancer growth, and the exact role of individual cytokines remain unclear. In this part, we focused on the role of IL-4, IL-10 and IL-13 on procathepsin D-stimulated proliferation of breast cancer cells. Our results clearly showed that only ER⁺ breast cancer cells responded to the presence of all three tested cytokines by proliferation, ER⁻ cells were resistant to the addition of cytokines (Figure 3). As addition of anti-procathepsin D antibodies blocked the growth potentiation of cancer cells, we can conclude that addition of these cytokines resulted in stimulation of synthesis and/or release of procathepsin D. This conclusion was further supported by findings of procathepsin D in culture supernatants of cells incubated with cytokines. These data are important for further attempts to inhibit procathepsin D production and secretion (Voburka et al. 2002). Peptide analogs employing D-amino acids and appropriate monoclonal antibodies have been prepared in July 2002, but due to the expiration of the award, they have not been tested.

KEY RESEARCH ACCOMPLISHMENTS

- ❑ Demonstration that the binding site of the procathepsin D activation peptide is located in the 27 – 44 AA region
- ❑ Syntetic library demonstrated the exact binding site of the activation peptide
- ❑ Establishment of all cell lines transfected with vectors containg human procathepsin D cDNA
- ❑ Establishment of basic values for invasiveness and *in vivo* correlation between procathepsin D production and cell growth *in vivo*

- Demonstration that invasiveness of breast cancer cells can be blocked by inhibition of procathepsin D secretion
- Demonstration that application of anti-procathepsin D antibodies or anti-fragment antibodies raised against fragments

REPORTABLE OUTCOMES

ABSTRACTS

- I. **Vetvicka, V., Vetvickova, J., Fusek, M., Voburka, Z.:** Blocking of growth factor activity of procathepsin D inhibits human cancer, **Faseb J.**, Abstracts, p. A1000, 2000.
- II. **Vetvicka, V., Vetvickova, J., Fusek, M., Voburka, Z.:** Inhibition of breast cancer by the procathepsin D activation peptide, **EACR XVI -2000 Meeting**, May 2000, Halkidiki, Greece, Abstract page 88.
- III. **Vetvicka, V., Vetvickova, J., Voburka, Z., Fusek, M.:** Procathepsin activation peptide and its fragments in regulation of cancer growth, oral presentation, **13th International Symposium on Regulatory Peptides**, Cairns, Australia, October 22-26, 2000.
- IV. **Vetvicka, V., Vetvickova, J., Fusek, M., Voburka, Z.:** Procathepsin D activation peptide and human cancer, **AACR Meeting Molecular Biology and New Therapies in the 21st Century**, Maui, February 12-16, 2001.
- V. **Vetvicka, V., Vetvickova, J., Voburka, Z., Fusek, M.:** Procathepsin D stimulates the growth of human cancer – effect of cytokines. 92th Annual Meeting of American Association of Cancer Research, New Orleans 2001, p. 878
- VI. **Vetvicka, V.:** Procathepsin D stimulation of human breast cancer cell growth, **Era of Hope**, Orlando, 2002

PAPERS

1. **Vetvicka, V., Vetvickova, J., Fusek, M.:** Role of procathepsin D activation peptide in prostate cancer development. **Prostate**, 44: 1-7, 2000
2. **Vetvicka, V.; Benes, P.; Fusek, M.:** Procathepsin D in breast cancer: What do we know? Effects of ribozymes and other inhibitors, **Cancer Gene Ther.**, 9, 854-863, 2002.
3. **Voburka, Z.; Vetvicka, V.; Vetvickova, J.; Fusek, M.:** Cytokines affect procathepsin D-stimulated proliferation of breast cancer cells, **Anticancer Res.**, 22: 913-919, 2002.
4. **Benes, P.; Koelsch, G.; Dvorak, B.; Fusek, M.; Vetvicka, V.:** Detection of procathepsin D in rat milk, **Comp Biochem. Physiol. B Biochem. Mol. Biol.**, 133: 113-119, 2002

CONCLUSIONS

Based on results mentioned above, we have all reasons to believe that this project will be finished in successful and timely manner. Now when we established the exact binding site of activation peptide, we will focus our attention on Task 2 and isolation of the specific receptor.

As far as experiments in Task 3 are concerned, we already established that synthetic fragment corresponding to the 27-44 AA portion of activation peptide is responsible for binding of procathepsin D to the cancer cells. Subsequent experiments helped to locate the actual binding site to a region of 36-44 AA portion of the activation peptide. Further on, experiments using a library of synthetic peptides with a single amino acid substitutions helped establish the exact binding site on procathepsin D activation peptide.

In addition, we focused our attention on possible regulation of procathepsin D synthesis by cytokines. Various cytokines are involved in both cancer development and in defense against cancer growth, and the exact role of individual cytokines remain unclear. In this study, we focused on the role of IL-4, IL-10 and IL-13 on procathepsin D-stimulated proliferation of breast cancer cells. Our results clearly showed that only ER⁺ breast cancer cells responded to the presence of all three tested cytokines by proliferation, ER⁻ cells were resistant to the addition of cytokines. As addition of anti-procathepsin D antibodies blocked the growth potentiation of cancer cells, we can conclude that addition of these cytokines resulted in stimulation of synthesis and/or release of procathepsin D. This conclusion was further supported by findings of procathepsin D in culture supernatants of cells incubated with cytokines.

So far, all obtained data support the original hypothesis that procathepsin D significantly stimulates the growth and spreading of breast cancer cells. If this hypothesis is further confirmed by this research project, this project has very significant potential to be developed into preclinical trials leading toward a new, very specific treatment of human breast cancer. In addition, recent observations by our and other groups suggest that the role of procathepsin D in human cancer development is probably even more general than we originally believed. In such a case, this project might be even more important.

Personnel

Vaclav Vetvicka, Ph.D. – PI
Petr Benes, Ph.D. – Postdoctoral fellow
Jana Vetvickova – Technician

Appendices

Legend to the Figures

Figure 1

Comparison of the procathepsin D secretion in tissue culture supernatants of six different cell clones (MDA-MB-231) transfected with vector containing cDNA coding human activation peptide using Western blotting.

Figure 2

Comparison of the procathepsin D secretion in tissue culture supernatants of four cell clones (MDA-MB-231) transfected with vector containing cDNA coding human activation peptide. Using an ELISA assay employing anti-activation peptide antibodies, we compared the levels of procathepsin D secreted into tissue culture supernatants of transfected and parental cells. These data were confirmed by Western blotting.

Figure 3

The same cell clones were used for study of cell growth in vivo. Athymic nude mice were injected with 5×10^6 cells ip. Ten days after injection, the mice were sacrificed, tumor excised and evaluated. The color codes correspond to the clones showed in Figure 1.

Figure 4

The growth of human cancer cells MDA-MB-231 in athymic nude mice can be inhibited by anti-procathepsin D antibodies and antibodies against a AA 36-44 fragment, but not by control antibodies or an AA 1-27 fragment.

Figure 5

In similar experiment, repeated injections of anti-procathepsin D antibodies and/or anti AA 27-44 fragment protected not only against breast cancer cell growth, but also against secondary challenge with prostate cancer cells LNCaP.

Figure 6

Only fragment AA 27-44 supported growth of cancer cell in a manner similar to either procathepsin D or activation peptide. ZR-75-1 cell line was used in this particular experiment.

Figure 1

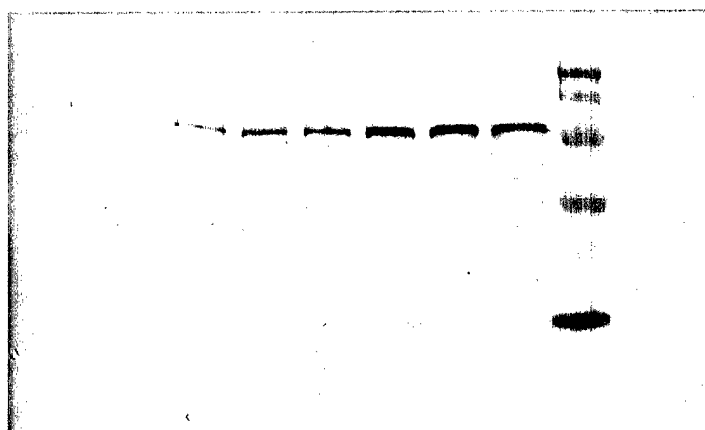


Figure 2

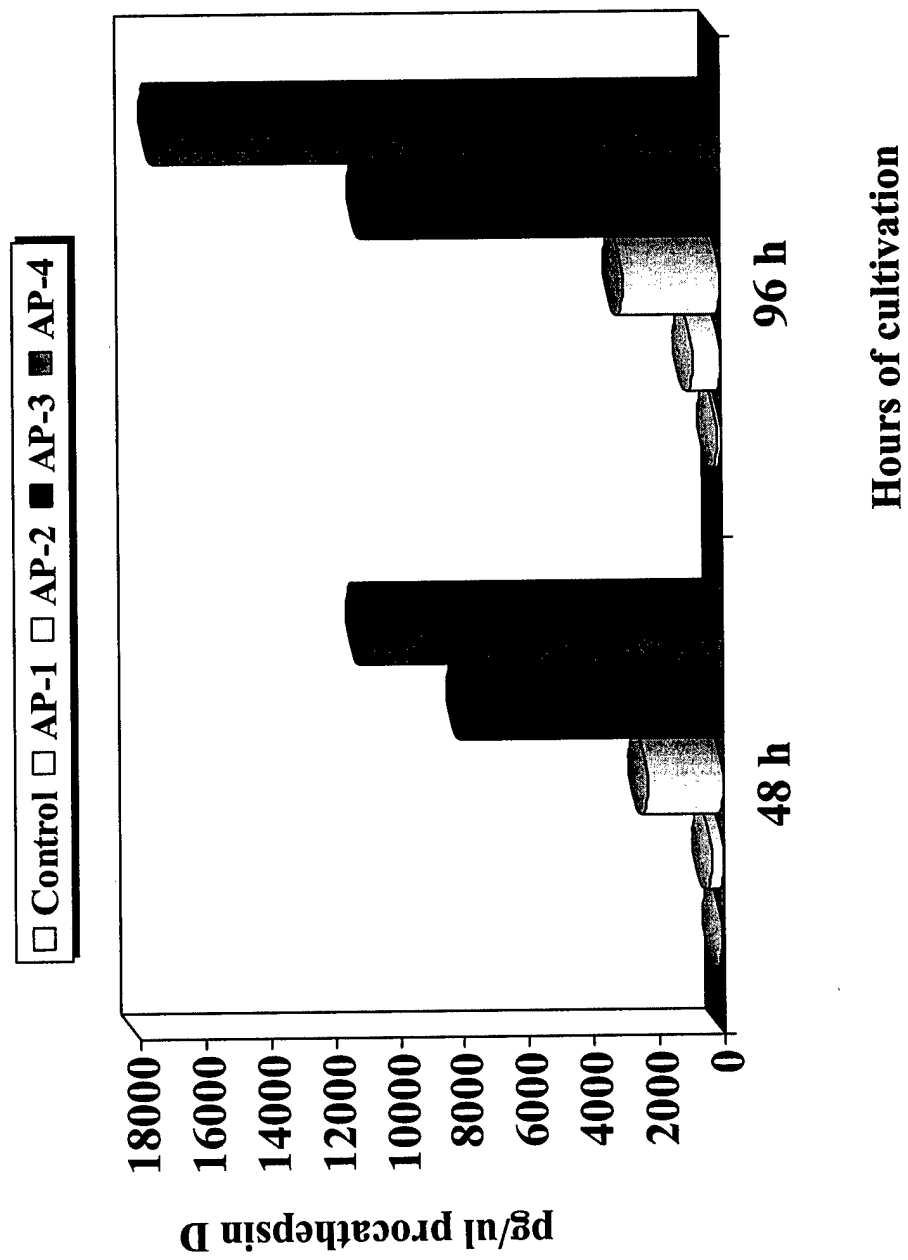
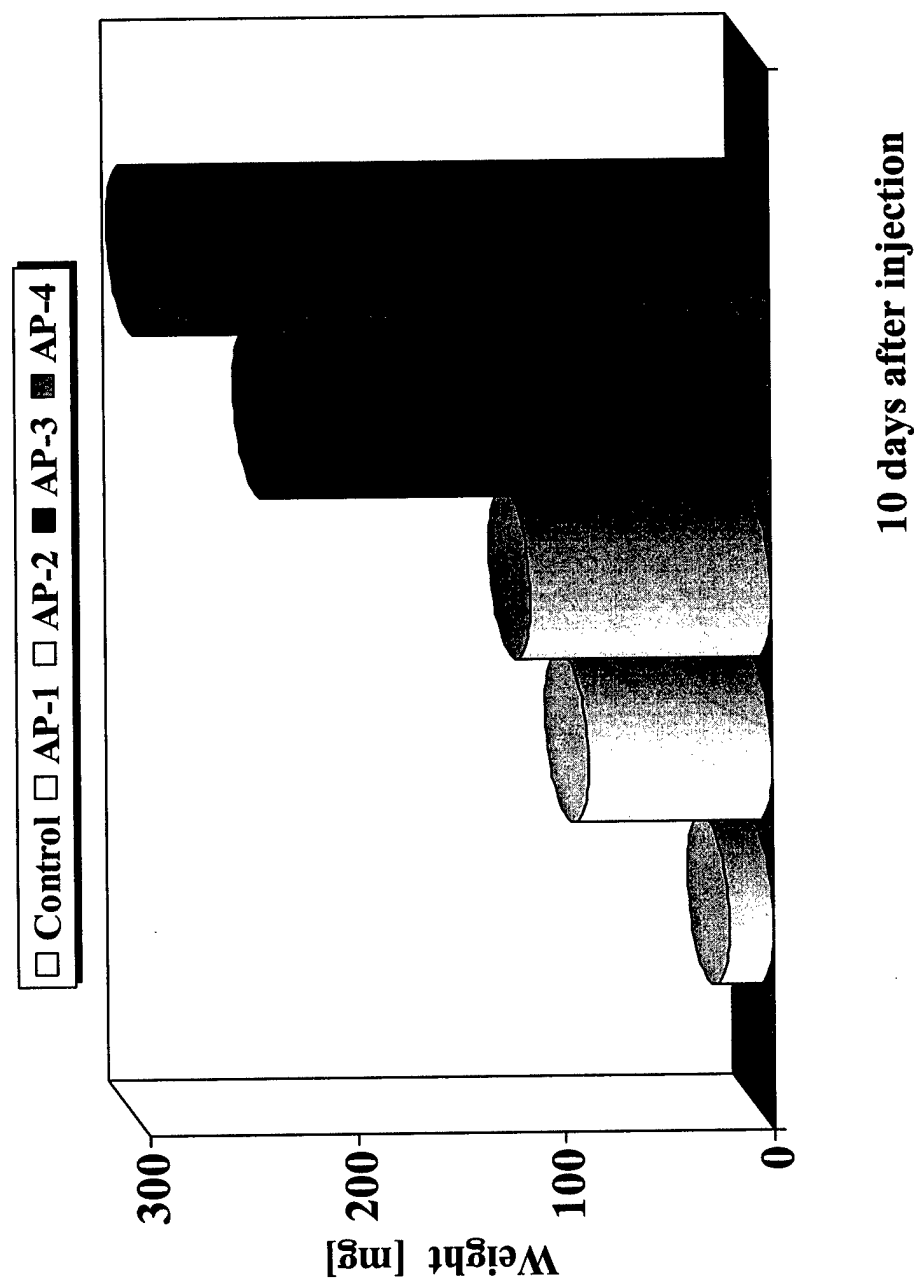


Figure 3



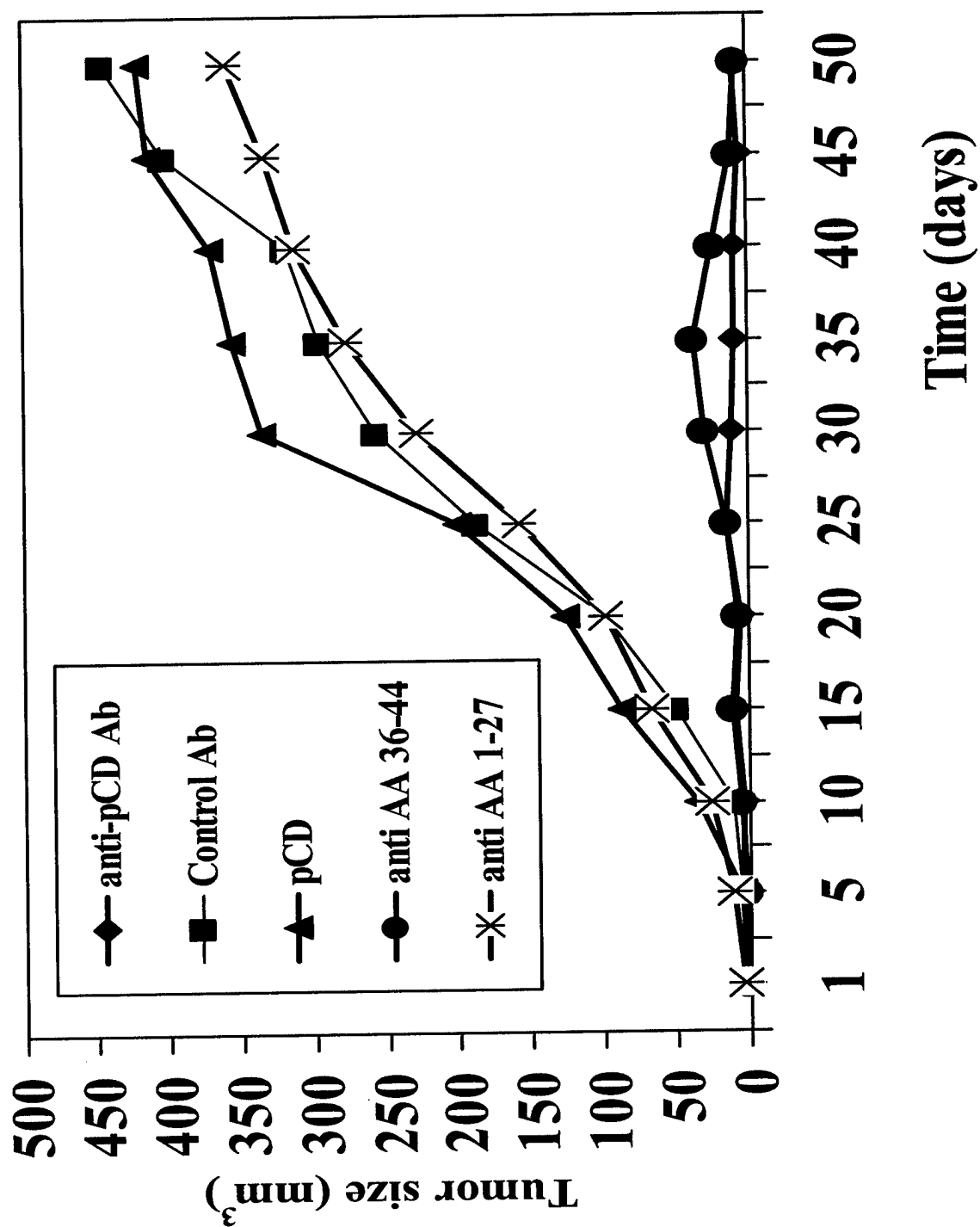
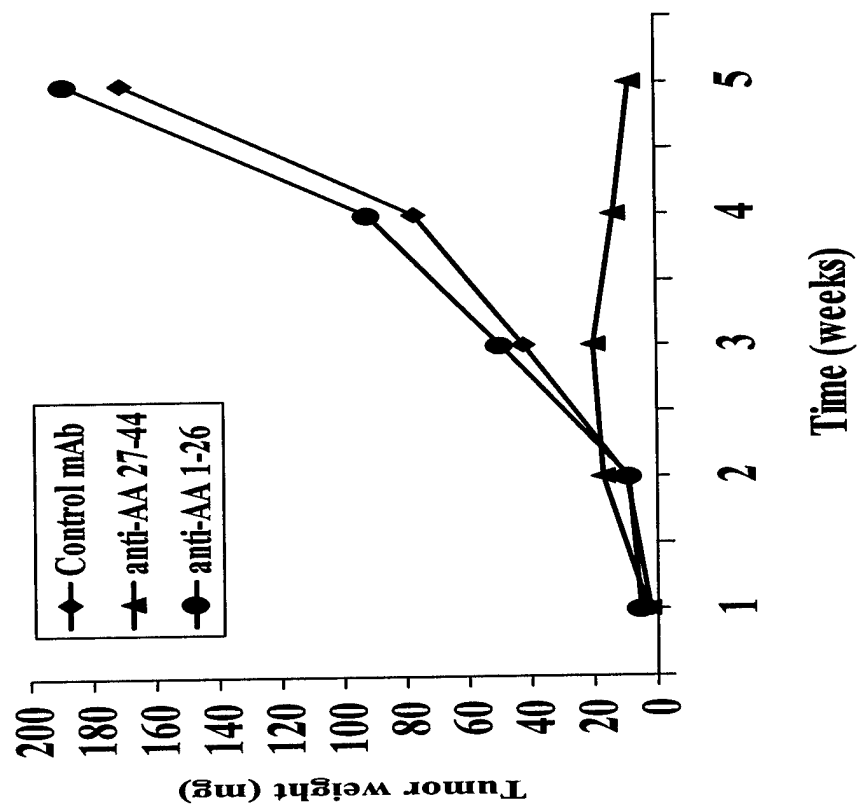


Figure 4

Figure 5

Secondary challenge with LNCaP cells



Inoculated with MDA-MB-231 cells

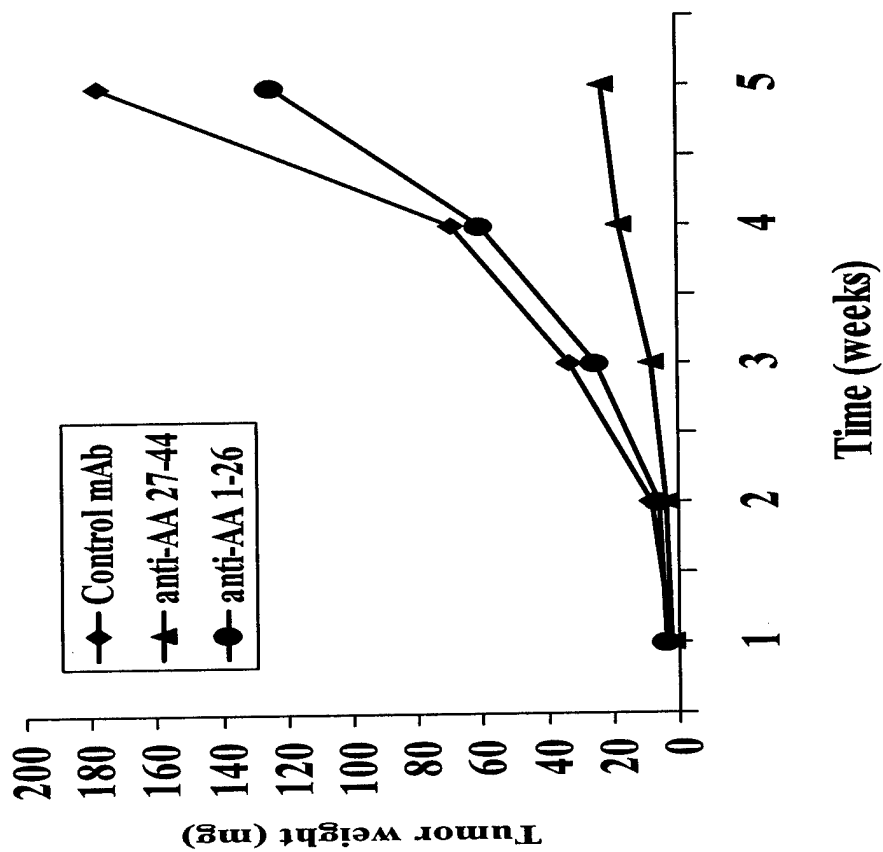
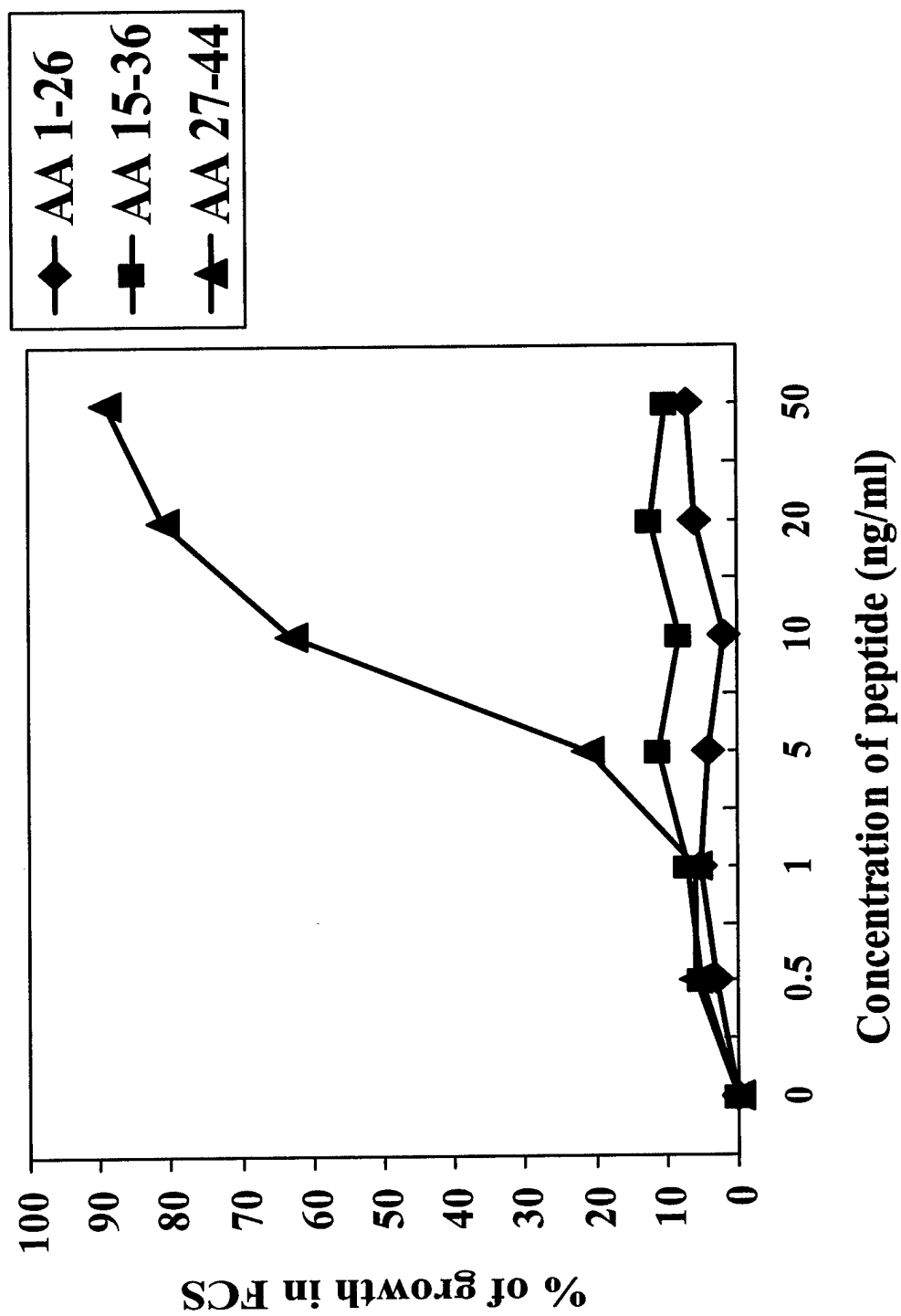


Figure 6



Role of Procathepsin D Activation Peptide in Prostate Cancer Growth

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BACKGROUND. Enzymatically inactive procathepsin D secreted from cancer cells has been confirmed to play a role in breast cancer development. We focused on prostate cancer and the role of activation peptide in mitogenic activity.

METHODS. Synthetic peptides and monoclonal antibodies raised against individual fragments of activation peptide were employed. Cell proliferation was measured by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay or by in vivo growth in nude mice.

RESULTS. We demonstrated that the growth factor activity of activation peptide is localized in amino-acid region 27-44. In addition, both anti-activation peptide and anti-27-44 peptide antibodies administered in vivo inhibited the growth of human prostate tumors in mice.

CONCLUSIONS. Based on these data, we hypothesize that the interaction of procathepsin D activation peptide with an unknown receptor is mediated by amino-acid sequence 27-44. This interaction leads in certain types of tumor to a proliferation and higher motility. Blocking of this interaction by antibodies or antagonists might be a valuable tool in prostate cancer inhibition. *Prostate* 44:1-7, 2000. © 2000 Wiley-Liss, Inc.

KEY WORDS: procathepsin; prostate cancer; proliferation; growth factors; activation peptide

INTRODUCTION

Cathepsin D (CD) and its enzymatically inactive zymogen procathepsin D (pCD) were originally established as prognostic markers in breast cancer [1]. In the last decade, increasing amounts of data have documented the direct involvement of CD and pCD in the growth of numerous types of cancer, including breast cancer [2-4], colorectal cancer [5], endometrial adenocarcinoma [6], ovarian cancer [7], hepatoma [8], and prostate cancer [9,10], suggesting that this system might be involved in general mechanisms of metastatic growth.

Our work on procathepsin D in human cancer originally showed that this enzymatically inactive precursor acts as an autocrine growth factor specific for breast cancer-derived cells [3,4]. Subsequent experiments demonstrated that the growth of breast cancer cells in vivo could be inhibited by anti-pCD antibodies [11]. The growth factor function of pCD was found to be mediated through a specific receptor expressed on

all eight human breast cancer cell lines examined. This receptor was found to be distinct from the well-known mannose-6-phosphate receptor, which has been shown to function as the CD receptor on most other systems. The parts of the procathepsin D molecule responsible for its mitogenic activity were localized near amino acids 27-44 of the activation peptide (APpCD) sequence [11]. In all our experiments, the mature enzyme CD had no role in metastasis.

Based on the currently suggested possible general role of pCD in cancer development, the last few years witnessed numerous studies searching for the involvement of CD and pCD in prostate cancer. At present, the role of pCD/CD in prostate cancer is unclear.

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Some studies found that the levels of CD correlate with the pathologic state, and some suggested that CD is a useful marker of prostate cancer progression [9]. Experiments using procathepsin D or its activation peptide showed that proliferation of all three human prostate cancer-derived cell lines was potentiated by the addition of either pCD or APpCD to the cultivation medium. In addition, prostate cancer cells were found to respond by proliferation to a number of paracrine factors including bombesin, somatostatin, or vasoactive intestinal peptide [12].

Even estrogen receptor-negative breast cancer cell lines are responsive to pCD [4], and probably there is no correlation with estrogen receptor status [13]. Recent papers showing the possible involvement of surrounding noncancerogenous cells in the secretion of pCD seem to suggest that in vivo, the presence of even a small population of pCD-secreting cells might have a dramatic effect on the proliferation of cells in surrounding tissues [14].

Based on these data, we decided to evaluate the hypothesis that a small 27–44 amino-acid fragment of the APpCD can serve as a growth factor for human prostate cancer cells, and that the anti-AP antibodies can inhibit the growth of prostate cancer in vivo. In conclusion, we propose that pCD via its activation peptide regulates the growth of prostate cancer by stimulation of cell proliferation, probably through specific interaction with a new, as yet unspecified receptor. Blocking of this interaction of pCD with its corresponding receptor might inhibit the development of prostate cancer. In addition, rapid screening for the receptor involved in pCD binding or for anti-AP antibodies in the blood of prostate cancer patients might be a future prognostic factor.

MATERIALS AND METHODS

Chemicals

RPMI 1640 medium, Iscove's modified Dulbecco's medium, HEPES, (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfuric acid]), MTT, E-TOXATE human cathepsin D, transferrin, glutaraldehyde, chondroitin sulfate, and porcine gelatin were obtained from Sigma Chemical Co. (St. Louis, MO); fetal calf serum was from Hyclone Laboratories (Logan, UT); pepstatin A and Protein A Sepharose were from Pharmacia (Pharmacia LKB Biotechnology, Piscataway, NJ), and pig pepsinogen A was from Worthington (Freehold, NJ).

Activation Peptide

The 44-amino-acid-long peptide corresponding to the activation peptide of pCD, the 28-amino-acid-long peptide (1–27 AA), and the 18-amino-acid-long pep-

tide (27–44 AA) corresponding to the fragment of AP were synthesized at the Institute of Organic Chemistry and Biochemistry (Academy of Sciences of the Czech Republic, Prague, Czech Republic). The purity of the activation peptide and its fragment was controlled using following methods: HPLC (High Pressure Liquid Chromatography) amino-acid analysis, and mass spectrometry. The HPLC method showed the purity to be more than 95%. The amino-acid analysis confirmed the amino-acid composition. Mass spectrometry results were in accordance with the proposed molecular structure. In addition to the techniques mentioned above, the purity of peptides was controlled also by N-terminal sequencing, using an automated system where the first nine N-terminal amino acids were in agreement with the designed structure.

Cell Cultivation

For growth experiments, cells were first incubated for 2 days in 0.1% FCS. The cells were harvested by centrifugation and washed six times in Iscove's modified Dulbecco's medium with HEPES buffer supplemented with glutamine, antibiotics, and 10 µg/ml of human transferrin [4]. Cells were seeded in 96-well tissue culture plates at a density of 5×10^4 cells/ml (150 µl/well) in the presence or absence of different concentrations of purified pCD or various substances tested in triplicate wells. After 5 days in culture, proliferation was evaluated using an MTT assay. The incorporation of MTT was stopped by the addition of 50 µl of 10% SDS in 0.01 N HCl, and the optical density (O.D.) of the well supernatants was measured 24 hr later at 570 nm, using the SLT ELISA reader (Tecan, Research Triangle Park, NC). All media were tested for endotoxin contamination and shown to contain <0.1 ng/ml Lipopolysaccharide (LPS) using the *Limulus* lysate test. Steroid-deprived cells were used in all experiments. The levels of procathepsin D in FCS at the concentrations used were below detection levels (data not shown). Similarly, the levels of estrogens in FCS were below 1 pg/ml. We repeated our experiments, using both charcoal-treated FCS and medium without phenol red, with identical results.

Human Cell Lines

Prostate cancer cell lines PC3, DU145, and LNCaP and breast cancer cell line ZR-75-1 were obtained from the American Tissue Culture Collection (ATCC, Manassas, VA). The cancer cell lines were grown in RPMI 1640 medium with HEPES buffer supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin in plastic disposable tissue culture flasks at 37°C in a 5% CO₂/95% air incubator.

Isolation of Procathepsin D

Human procathepsin D was isolated from the culture supernatant of human breast cancer cell line ZR-75-1, as described earlier [15]. Briefly, a two-step procedure was used. In the first step, immunoaffinity chromatography was used, with anti-activation peptide antibodies attached to Protein A Sepharose. In the second step, FPC (Fast Protein Chromatography) chromatography using a Mono-Q column (Pharmacia LKB Biotechnology) and 20 mM TRIS (pH 7.2) was used.

Animals

Athymic nu/nu BALB/c mice were purchased from Jackson Laboratory (Bar Harbor, ME).

Antibodies

Anti-human factor I OX-21 IgG₁ mAb was described elsewhere [16]. Anti-human cathepsin D IgG_{2a} mAb was purchased from Calbiochem (La Jolla, CA). The anti-27-44 AA fragment of the activation peptide was described previously [17]. Each mAb (except anti-cathepsin D) was isolated from ascites fluid by 50% ammonium sulfate precipitation followed by Mono-Q anion exchange chromatography.

Preparation of Biodegradable Microspheres

Biodegradable gelatin microspheres were prepared as described [11,18]. Briefly, 5% gelatin solution was mixed with mAbs diluted in 0.2% chondroitin sulfate at room temperature. Coacervation was achieved by rapidly adding 5 mg of gelatin solution to 5 ml of the stirred chondroitin sulfate solution. The microspheres were cross-linked by the addition of 50 μ l of 25% glutaraldehyde solution. After 30 min of incubation, 6 ml of ethanolaminated-HCl were added. The microspheres were then washed six times with phosphate-buffered saline.

Tumor Cell Growth in Mice

Human prostate tumors were generated by injecting athymic nude mice with 5×10^5 LNCaP cells subcutaneously. After 10-14 days, the mice were checked for tumor development, and only mice with palpable tumors were subsequently injected intravenously with biodegradable gelatin microspheres containing 100 μ g of either anti-pCD mAb, anti-fragment mAbs, or irrelevant anti-human factor I IgG₁ mAb (three injections 2 weeks apart). Mice were sacrificed at various time intervals, and tumor size was evaluated by weighing the tumors.

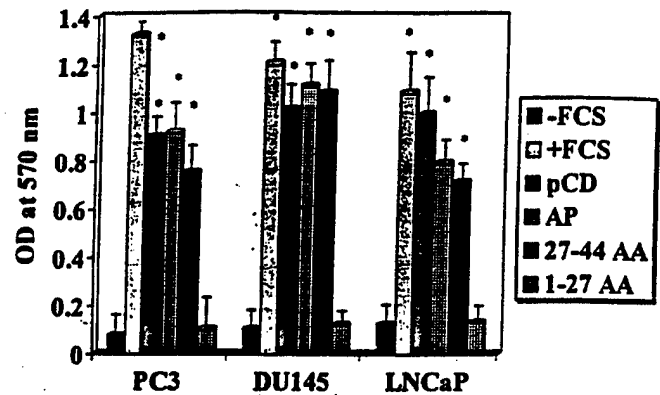


Fig. 1. Growth of human prostate cancer cell lines in serum-free medium containing 50 ng/ml of activation peptide (AP), 10 ng/ml of procathepsin D (pCD), 50 ng/ml of 1-27 AA fragment (1-27 AA), or 50 ng/ml of 27-44 AA fragment (27-44 AA) of the activation peptide. The results from growth in the serum-free medium (-FCS) and in control medium supplemented with FCS (+FCS) are given for comparison. The results represent the mean \pm SD of three independent experiments. *Significant differences from control (-FCS) values at $P \leq 0.05$.

Statistical Evaluation

Statistical significance of the differences of the means was evaluated by Student's *t*-test.

RESULTS

Cell Culture

The results showing the effect of 50 ng/ml of activation peptide, 10 ng/ml of procathepsin D, or 50 ng/ml of 27-44 AA fragment of the activation peptide on the proliferation of three different prostate cancer-derived cell lines are shown in Figure 1. Data presented in Figure 1 were obtained after 5 days of incubation instead of the 7 days usually used, in case of more slowly growing breast cancer-derived cells. Proliferation was measured by the incorporation of MTT by cells. The data show that the proliferation of cells was increased in the same way by pCD, activation peptide, or a 27-44 AA fragment of the activation peptide. The proliferative activity of these molecules for prostate cancer cells was dose-responsive, with maximum proliferative activity at a concentration of 10 ng/ml (data not shown). Our previous experiments showed that this dose was the optimal dose both on breast cancer cells [4] and in a prostate cancer cell model [10].

Determination of Possible Involvement of Cathepsin D

Experiments using the enzymatically inactive zymogen of CD always raise the possibility that the en-

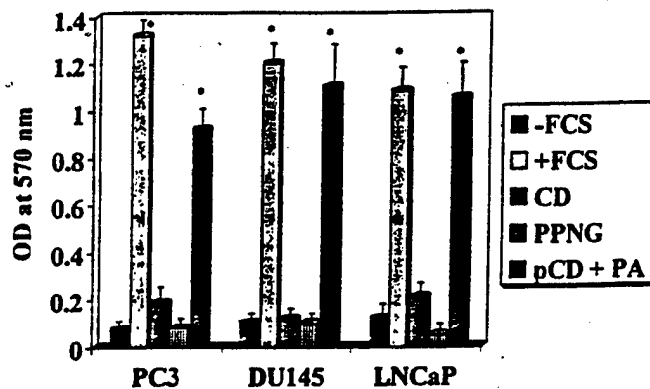


Fig. 2. Growth of human prostate cancer cell lines in serum-free medium is not supported by 2 nM human cathepsin D (CD) or 20 nM pig pepsinogen A (PPNG). Similarly, growth-stimulating activity of procathepsin D (pCD) was not inhibited by 1 μ M pepstatin A (PA). The results from growth in serum-free medium (-FCS) and in control medium supplemented with FCS (+FCS) are given for comparison. The results represent the mean \pm SD of three experiments. *Significant differences from control (-FCS) values at $P \leq 0.05$.

zymatically fully active CD might be formed during the duration of the experiment. We decided to address this possibility by using the mature human CD instead of pCD (Fig. 2). However, CD failed to stimulate the proliferation of prostate cancer cells. In addition, we used pepstatin A, a strong inhibitor of cathepsin D, with K_i at the picomolar level [19], to test the possible involvement of the proteolytic activity of CD on the observed mitogenic function. All these experiments showed that the enzymatic activity of CD had very little influence on the growth-factor activity of pCD. Similarly, no proliferative activity was observed for pig pepsinogen A as a control molecule. Pepsinogen was included as a control protein with a similar overall structure.

27-44 Amino-Acid Fragment of Activation Peptide

Despite these demonstrations that the proteolytic activity of the mature enzyme is not involved in the described effects, we decided to use only synthetic peptides in all subsequent experiments. As shown in Figure 1, there are no significant differences between the activity of the complete molecule of pCD or its activation peptide. Even a fragment of the activation peptide showed a similar level of activity. A strong inhibition of the mitogenic activity of activation peptide was observed when monoclonal antibodies raised against the activation peptide of pCD or against the 27-44 AA fragment of the activation peptide were used (Fig. 3). The specificity of these two antibodies used in this experiment was described previously [17];

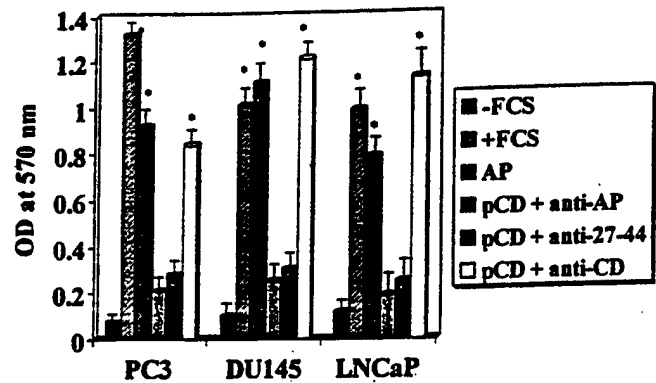


Fig. 3. Stimulation of human prostate cancer cell line growth in serum-free media and its inhibition by various monoclonal antibodies. Procathepsin D (10 ng/ml) was inhibited by 1 μ g/ml of anti-activation peptide IgG (anti-AP), anti-27-44 AA fragment of activation peptide IgG (anti-27-44), or anti-human cathepsin D IgG (anti-CD). The results from growth in serum-free medium (-FCS) and in control medium supplemented with FCS (+FCS) are given for comparison. The results represent the mean \pm SD of three experiments. *Significant differences from control (-FCS) values at $P \leq 0.05$.

briefly, anti-pCD antibodies recognize epitopes contained within the activation peptide only, while anti-CD antibodies interact with epitopes of the mature enzyme and not with the activation peptide. Similar to our observation in a breast cancer model, this inhibition of the mitogenic effect observed for anti-AP antibodies was specific for pCD, and these antibodies had no effect on the function of other known mitogens [3].

In Vivo Treatment

The previously mentioned experiments were followed by a final set of experiments where we focused on the growth-stimulating activity of pCD in an in vivo model in which both anti-activation peptide and anti-27-44 AA antibodies were shown to reverse cancer development. Cells of the human prostate cancer cell line LNCaP were injected s.c. into athymic nude mice. After evaluation for development of tumor mass, mice were injected with biodegradable gelatin microspheres containing 100 μ g of antibodies (day 0). Preliminary experiments using both breast cancer and prostate cancer cells revealed that 100 μ g of antibodies per injection is the optimal dose. No significant difference between 100, 200, and 300 μ g of antibodies was observed (unpublished results). Four mice per experimental group were killed at each time interval, and the tumor mass was carefully extracted and cleaned of all surrounding tissue. Inhibition and regression of tumor size were determined by weight. The first tumor mass was palpable approximately 10 days after the initial injection. However, at this time period the ac-

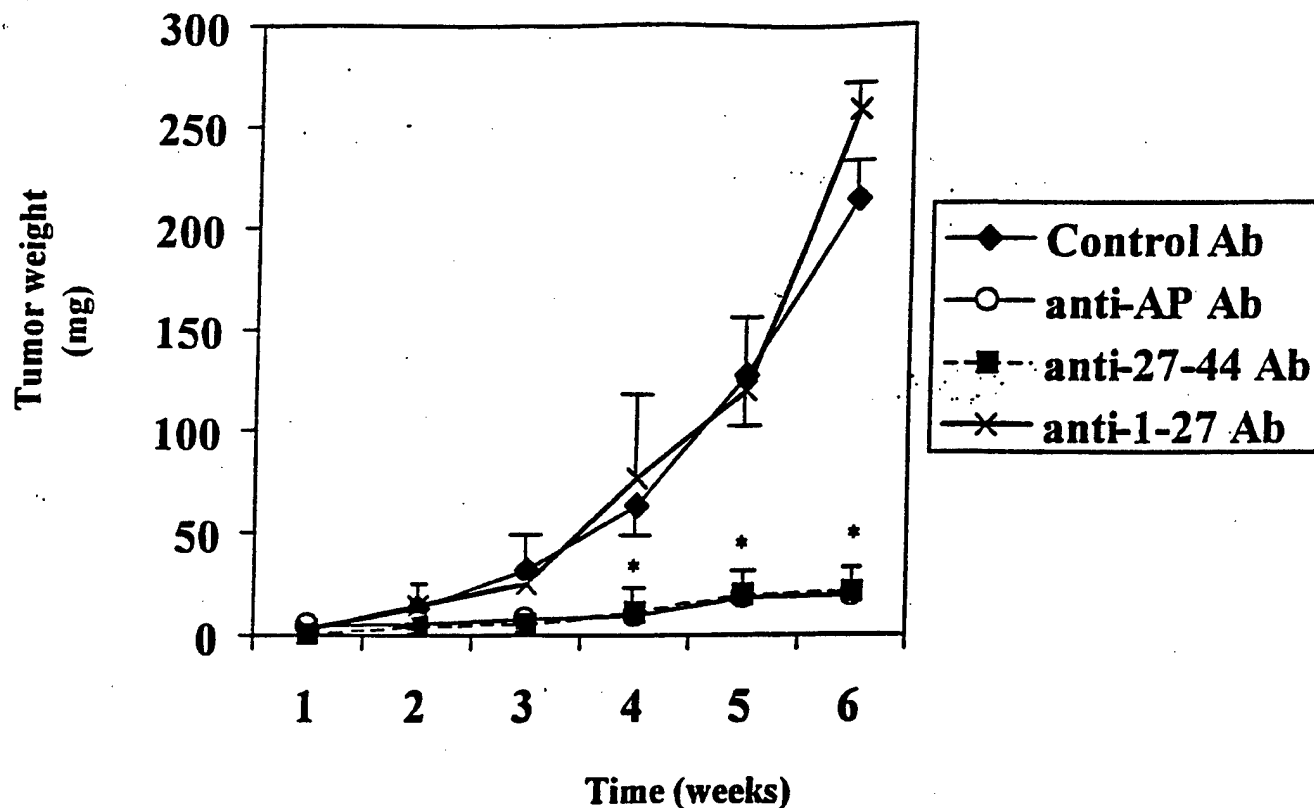


Fig. 4. Inhibition of LNCaP cell tumor growth in athymic mice by treatment with anti-activation peptide (anti-AP Ab), anti-1-27 AA fragment (anti-1-27 Ab), anti-27-44 AA fragment (anti-27-44 Ab), or control anti-factor I (Control Ab) antibodies. See Materials and Methods for experimental details. Results represent mean \pm SD of 4 mice per time interval. *Significant differences from control (Control Ab) values at $P \leq 0.05$.

tual weight of tumors is very low, and most of the mass is formed by small granulomas. Figure 4 shows that mice treated with either anti-activation peptide or anti-27-44 AA antibodies had greatly reduced tumor growth. On the other hand, irrelevant anti-factor I antibodies or the antibodies raised against 1-27 AA fragment of the activation peptide (previously shown to be without any significant biological activity [11,17]) had no effect on cancer growth. The antibody-mediated protection from an induced breast cancer was almost complete for the whole tested interval of 6 weeks.

DISCUSSION

The role of cathepsin D and its enzymatically inactive precursor procathepsin D in cancer development has recently attracted considerable attention. Besides their more common involvement in human breast cancer [11,20-23], the significance of procathepsin D and cathepsin D levels in various tumors such as endometrial adenocarcinoma [6], prostate cancer [24], colon carcinoma [25], laryngeal tumors [26], ovarian cancer [7], and carcinoma of thyroid tissue [27] has been dem-

onstrated, suggesting that this system might be involved in the metastatic growth of several other cancer cell types. Numerous studies have demonstrated that this proenzyme is overexpressed and secreted from several types of cancer (such as breast, melanoma, and ovary). In addition, elevated anti-pCD antibodies have been found in plasma of breast [28] or ovarian [7] cancer patients. Numerous papers showed both the in vitro effects of pCD on cancer cell proliferation [3,10,29,30] as well as effects in vivo [11]. Furthermore, induced overexpression of pCD significantly stimulated metastasis in experimental models of mice injected with a rat cancer cell line transformed with human procathepsin D cDNA [30,31].

The synthetic peptides were prepared with the highest possible purity. Nevertheless, the possibility that some of the observed biological effects might be caused by some impurities cannot be completely overlooked. On the other hand, we used extremely low concentrations; thus, it is not probable that the potential impurities might be active in 1 ng/ml concentrations. In addition, we used procathepsin D isolated from the tissue culture supernatant by means of affinity chromatography with virtually identical results.

Furthermore, we believe that the use of synthetic peptides eliminates the effects of possible contaminants much more completely than when biologically isolated material is used.

This paper focused on the hypothesis that the mitogenic growth factor activity of pCD is mediated by its activation peptide [11]. This possibility is generally supported by several functions which the activation peptide was found to play within the family of aspartic proteinases. For example, in the case of pCD it is speculated that a structure within the activation peptide is involved in targeting to lysosomes (in addition to classical mannose-6-phosphate pathways) [32,33]. No inhibition of activation peptide binding or activities by either mannose-6-phosphate, anti-mannose-6-phosphate receptor antibodies, or soluble mannose-6-phosphate receptor was found [11]. As direct mitogenic action has been already demonstrated, the aim here was to determine which parts of the pCD are responsible for receptor activation. Direct mitogenic action has been already demonstrated using several models, including breast and prostate cancer [3,4,10]. In addition, the activation peptide of the pCD molecule was found to be responsible for growth factor activity [11]. Therefore, the aim here was to determine if the activation peptide is similarly involved in prostate cancer proliferation. Additional attention was focused on the question of which amino-acid residues in the activation peptide might be responsible for activation of the receptor involved in this reaction. Finally, to check directly the possibility that pCD is involved significantly in the progression of prostate cancer in vivo, we injected athymic nude mice with a prostate cancer cell suspension. Two weeks later, the mice were treated with biodegradable microspheres containing anti-activation peptide or anti-27-44 AA fragment antibodies. From the results we could conclude that the prolonged administration of these antibodies protected nude mice for up to 6 weeks (the entire tested interval). Previous results showed that elevated secretion of pCD increased the metastatic potential of transfected cells [34]. Our experiments demonstrate even more directly the involvement of pCD in prostate cancer development, and identify the activation peptide as a new potential target for suppression of human prostate cancer.

CONCLUSIONS

The presented data confirm our previous observation [10] that human prostate cancer cells respond to both pCD and its activation peptide by a significant increase of proliferation. The contributions of various enzymes and their zymogens to prostate cancer development were recently the subject of numerous papers

[35,36]. In addition, functional expression cloning identified CD as a candidate gene for prostate cancer progression [37]. The data presented in our paper seem to be in agreement with the so-called Fusek and Vetvicka model [38], where pCD does not act as the inactive precursor of the enzyme, but as a ligand for triggering a transmembrane receptor. We propose that the mechanism of the mitogenic function of pCD relies on a specific structure of the activation peptide, most probably inside its 27-44 AA fragment, and its interaction with a cell membrane receptor present on the surface of at least breast and prostate cancer cells. The experiments attempting to isolate and characterize this new receptor are currently in progress.

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Procathepsin D in breast cancer: What do we know? Effects of ribozymes and other inhibitors

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Procathepsin D (pCD) is a major secreted glycoprotein in some human breast and other cancer cell lines. Several groups proposed that pCD served as a growth factor for these cell lines. Secreted pCD has been demonstrated in tissue section, tissue culture supernatants, carcinoma cytosols, and nipple aspirates. Moreover, several clinical studies suggested a potential role for this molecule in metastasis because its concentration in primary tumors correlated with an increased incidence of tumor metastases. In this paper, the effects of pCD were evaluated by proliferation *in vitro* and by mouse studies *in vivo*. Subsequent flow cytometry experiments showed the specificity of pCD binding to cancer cells. Cell cultivation showed that addition of either pCD or its activation peptide stimulates growth of cancer cells. These effects can be inhibited both *in vitro* and *in vivo* by anti-pCD antibodies. In addition, production of pCD can be inhibited by specifically designed ribozymes. This paper is focused on mitogenic effects of pCD, which seem to involve interaction of the activation peptide with as yet unidentified receptor. Different mechanisms by which pCD could promote development and spread of cancer cells are discussed.

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Keywords: procathepsin D; cathepsin D; breast cancer; receptor

Cathepsin D (CD; E.C.3.4.23.5) is a soluble lysosomal aspartic proteinase. It is synthesized in the endoplasmic reticulum as a preprocathepsin D.^{1,2} After removal of the signal prepeptide by signal peptidases and having a mannose-6-phosphate tag, procathepsin D (pCD) is recognized by a mannose-6-phosphate receptor (M6P-R). The M6P-R targets pCD to lysosomes. Upon entering into an acidic lysosome, the single-chain pCD (52 kDa) is activated to CD and subsequently to a mature two-chain CD (31 and 14 kDa, respectively).³ The two M6P-Rs involved in the lysosomal targeting of pCD are expressed both intracellularly and on the outer cell membrane. The glycosylation followed by phosphorylation is believed to be crucial for normal intracellular trafficking. Under physiologic conditions, pCD is sorted to the lysosomes and neither pCD nor CD is secreted. The fundamental role of CD is to degrade intracellular and internalized proteins.⁴ CD has been suggested to take part in antigen processing⁵ and in enzymatic generation of peptide hormones. The tissue-specific function of CD seems to be connected to the processing of prolactin. Rat mammary glands use this enzyme for the formation of biologically active fragments of prolactin.⁶ CD is functional in a wide

variety of tissues during their remodeling or regression and in apoptosis.⁷

In recent years, an increased amount of data has documented an important role of CD and pCD in cancer development.^{8–12} Increased levels of CD were first reported in several human neoplastic tissues in the mid-1980s.^{13,14} Subsequent studies employing several different approaches such as immunohistochemistry, *in situ* hybridization, cytosolic assay, Northern blot, and Western blot analyses have indicated that in most breast cancer tumors, pCD is overexpressed 2- to 50-fold.¹⁵ The overexpression was demonstrated both at the mRNA and protein levels. Vignon et al.¹⁶ first proposed that pCD secreted from breast cancer cell lines might serve as an autocrine growth factor and promote the cancer cell growth. These findings generated intense research in a possible role for CD in neoplastic processes. This research is focused into two major subjects: (a) use of CD (respectively, pCD) levels for diagnostic/predictive purposes; and (b) study of the molecular mechanism of pCD involvement in biological processes.

The synthesis of CD is controlled by steroid hormones. Progesterone and its derivatives increase the rate of uterine CD synthesis,¹⁷ and in breast cancer cell lines, CD expression is regulated by estrogens¹⁸ that interact at the promoter level.¹⁹ In ER⁺ cell lines, pCD is secreted only after estrogen stimulation. The estrogen stimulation of pCD secretion from breast cancer cells can be specifically inhibited by a specific estrogen inhibitor 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.²⁰ Conversely, it is secreted constitutively in

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ER⁻ cell lines.²¹ The mechanism of regulation of expression has been studied extensively and DNA sequences responsible for this regulation have been determined.^{19,22}

A strong predictive value was found for CD concentrations in breast cancer as well as many other tumor types.^{15,23,24} Subsequently, it has been recommended that levels of CD be monitored as a prognostic indicator in breast cancer and used independent of classical prognostic parameters such as tumor size, histological grade, lymph node status, and steroid receptor expression.²⁵⁻²⁷ The significance of pCD and CD levels in other tumors such as endometrial adenocarcinoma,^{28,29} colon carcinoma,³⁰ colorectal carcinoma,³¹ oral cancer,³² laryngeal tumors,^{33,34} head and neck squamous cell carcinoma,³⁵ carcinoma of thyroid tissue,³⁶ prostate tumors,^{37,38} melanoma,^{8,39} gastric cancer,⁴⁰ and ovarian cancer,^{41,42} has also been demonstrated, suggesting that this system might be involved in the metastatic growth of numerous other cancer cell types.

The second part of the research, which focused on the mechanism of CD/pCD involvement in breast cancer development, remains unclear. The situation is complicated by the fact that, at any given time, three different components of CD are present in breast tumor tissue: (a) pCD, (b) mature enzyme CD (which is present often in two forms — single chain and double chain), and (c) differently posttranslationally modified pCD. The authors very often do not discriminate between these forms when describing their results. For example, the antibodies that have been used for visualization of CD in tissue samples react with both CD and pCD. From currently available data, it is clear that when using Western blotting, which distinguishes pCD and other forms by molecular weight, most of these molecules are in the form of pCD. It has never been demonstrated that the secreted pCD is converted to the mature enzyme in an extracellular milieu, which is not sufficiently acidic. Despite numerous studies suggesting the involvement of mature CD,⁴³ this direct proteolytic involvement in the invasiveness of breast cancer cells has never been demonstrated.^{44,45} For a review and more details about the role of CD in cancer, see Ref. [46]. For the understanding of pCD/CD role in cancer development, we see as crucial the discrimination between the two forms — pCD and CD.

In this paper, we focused on the mechanism of pCD in the regulation of cancer growth and development by both overview of the latest results achieved by our group and by discussing the significance of the pCD in several types of cancer.

Materials and methods

Chemicals

RPMI 1640 medium, Iscove's modified Dulbecco's medium, HEPES, MTT, E-TOXATE human CD, transferrin, bovine CD, and M6P were obtained from Sigma (St. Louis, MO); fetal calf serum was from Hyclone Laboratories (Logan, UT); complete Freund adjuvant and pig pepsinogen A from Worthington (Freehold, NJ); and pepstatin A and Protein A Sepharose from Pharmacia LKB Biotechnology (Piscataway, NJ).

Synthetic peptides

The 44-amino-acid-long peptide corresponding to the activation peptide of pCD, the 26-amino-acid-long peptide (1–26 AA), the 22-amino-acid-long peptide (15–36 AA), and the 18-amino-acid-long peptide (27–44 AA) corresponding to the fragment of AP were synthesized in the Institute of Organic Chemistry and Biochemistry (Academy of Sciences of the Czech Republic, Prague, Czech Republic). The purity of the activation peptide and its fragment was controlled using following methods: HPLC, amino acid analysis, and mass spectrometry. The HPLC method showed the purity to be more than 95%. The amino acid analysis confirmed the amino acid composition. Mass spectrometry results were in accordance with the proposed molecular structure. In addition to techniques mentioned above, the purity of peptides was controlled also by N-terminal sequencing using an automated system where the first nine N-terminal amino acids were in agreement with the designed structure.

Cell cultivation

For growth experiments, cells were first incubated for 2 days in 0.1% FCS. The cells were harvested by centrifugation and washed six times in Iscove's modified Dulbecco's medium with HEPES buffer supplemented with glutamine, antibiotics, and 10 µg/mL human transferrin. Cells were seeded in 96-well tissue culture plates at a density of 5×10^4 cells/mL (150 µL/well) in the presence or absence of different concentrations of purified pCD or various substances tested in triplicate wells. After 5 days in culture, the proliferation was evaluated using an MTT assay. The incorporation of MTT was stopped by the addition of 50 µL of 10% SDS in 0.01 N HCl and the OD of the well supernatants was measured 24 hours later at 570 nm using SLT ELISA reader (Tecan, Research Triangle Park, NC). All media were tested for endotoxin contamination and shown to contain <0.1 ng/mL LPS using the *Limulus* lysate test. Steroid-deprived cells were used in all experiments. The levels of pCD in FCS at the concentration used were below detection levels (data not shown).

Human cell lines

Human breast cancer cells lines ZR-75-1 and MCF-7, human B lymphoblastoid cell line Raji, human breast cell line HBL-100, and prostate cancer cell line LNCaP were obtained from the American Tissue Culture Collection (ATCC; Manassas, VA). Breast cancer cell lines MDA-MB-231, MDA-MB-483, and MDA-MB-436 were obtained from Dr Ceriani of the John Muir Cancer and Aging Research Institute (Walnut Creek, CA). The T lymphoblastoid cell line 8402 was obtained from The Tissue Culture Facility of the Lineberger Cancer Research Center of the University of North Carolina-Chapel Hill (Chapel Hill, NC). The cancer cell lines were grown in RPMI 1640 medium with HEPES buffer supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin in plastic disposable tissue culture flasks at 37°C in a 5% CO₂/95% air incubator.

Antibodies

Monoclonal antibodies against activation peptide and its fragments were described previously.⁴⁷ IgG was isolated from ascites fluid by 50% ammonium sulfate precipitation followed by Mono-Q anion exchange chromatography. Monoclonal anti-CD antibodies were purchased from Calbiochem-Novabiochem (La Jolla, CA). Control MOPC-21 IgG antibody was purchased from Sigma. Goat anti-human 300 kDa M6P receptor antibody was donated by Dr K von Figura and by Dr T Bräulke (Georg-August University, Göttingen, Germany); rabbit antihuman M6P receptor antibody was donated by Dr S Kornfeld (University of Washington, St. Louis, MO). Soluble Ca-independent M6P-IGF II receptor was donated by Dr P Lobel (Center for Advanced Biotechnology and Medicine, Piscataway, NJ).

Isolation of pCD

Human pCD was isolated from the culture supernatant of human breast cancer cell line ZR-75-1 as described earlier.⁴⁸ Briefly, a two-step procedure was used. In the first step, immunoaffinity chromatography was used with antiactivation peptide antibodies attached to Protein A Sepharose. In the second step, FPC chromatography, using a Mono-Q column and 20 mM Tris (pH 7.2), was used.

Ribozymes

The RNase H mapping in cell extract was carried out by a slightly modified procedure as described by Scherr and Rossi.⁴⁹ Approximately 8×10^7 cells were pelleted and washed twice in PBS. The pellets were resuspended in hypotonic swelling buffer (7 mM Tris-HCl, pH 7.5, 7 mM KCl, 1 mM MgCl₂, 1 mM β -mercaptoethanol), incubated for 10 minutes on ice, transferred to a Dounce homogenizer, followed by addition of 1/10 of the final volume of neutralizing buffer (21 mM Tris-HCl, pH 7.5, 116 mM KCl, 3.6 mM MgCl₂, 6 mM β -mercaptoethanol). The homogenate was centrifuged at $20,000 \times g$ for 10 minutes at 4°C. The supernatants will be stored at -70°C in hypotonic buffer containing 45% glycerol. The RNase H-mediated cleavage experiments were carried out in a total volume of 30 liters, containing 20 liters of cell extract, 1 mM DTT, 20–40 U RNase inhibitor (Promega), and 50 nM of the various antisense oligodeoxyribonucleotides corresponding to ribozyme target sequences identified by MFOLD program. Sense oligodeoxyribonucleotide will serve as a control. This mixture was incubated for 5–10 minutes at 37°C followed by digestion with DNase I for 45 minutes and phenol extraction and ethanol precipitation. Reverse transcription was performed according to the manufacturer's protocol (Life Technologies, Carlsbad, CA) using 50 ng of random hexamer primer and 10 U of Moloney murine leukemia virus reverse transcriptase.

Different aliquots of the RT reaction were then amplified using pCD primers (5'-CCAGTACTACGGGGAGATTG-3' and 5'-CCATAGTGGATGTCAAACGA-3') and β -actin-specific primers (5'-TGCTATCCAGGCTGTGCTAT-3' and 5'-TTCCAGTTTTTAAATCCTGAGTC-3') for 25 cycles. Sequences of oligodeoxynucleotides that show the highest

potential in reduction of pCD mRNA in cell extracts were chosen as target sites for ribozymes. DNA sequences coding for ribozymes were synthesized by PCR using overlapping oligonucleotide primers. The PCR primers were designed in such a way that the corresponding PCR products will contain the T7 promoter sequence at their 5' ends. PCR products were fractionated through 3% composite agarose gel (2% NuSieve, 1% Seakem) (FMC, Rockland, ME), electroeluted, and purified with phenol/chloroform. The DNA sequence corresponding to target pCD mRNA sequence cloned to pcDNA3 vector (Invitrogen, Carlsbad, CA) was kindly provided by Dr John Chirgwin.

Cleavage activity of all ribozymes and control ribozymes was tested by *in vitro* cleavage assay. Ribozyme RNA, control RNA, and target pCD RNA were prepared by *in vitro* transcription reaction using RiboScribe RNA Probe Synthesis Kit (Epicentre, Madison, WI) according to the manufacturer's instructions. Cleavage reaction was performed by the method described by Biegelman et al.⁵⁰ PCR products were digested with appropriate restriction endonucleases (New England Biolabs, Beverly, MA) and ligated into pCI-neo and pH Apr-1-neo vectors. These vector constructs were transformed to competent *Escherichia coli* DH5 (Gibco, Carlsbad, CA). Large-scale vector DNA with cloned ribozyme and control sequences were then prepared using an EndoFree Plasmid Maxi Kit (Qiagen, Valencia, CA). All plasmid constructs were custom-sequenced (Invitrogen) to confirm the sequence and orientation of ribozyme templates.

Subsequently, human breast cancer cell lines were stably transfected by lipofection with either pH Apr-1-neo or pCI-neo vectors with cloned ribozyme and control sequences as mentioned above. The expression of ribozymes in transfected cells was screened as described by Dawson and Marini.⁵¹ The resulting PCR products were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining. The lengths of PCR products were compared with those predicted from vector ribozyme sequences. The expression of target pCD RNA in transfected cells was analysed using both the semiquantitative RT-PCR and Northern blot analysis.

Matrigel assay

For evaluation of the invasion across Matrigel layers, a commercial kit manufactured by Chemicol International (Temecula, CA) has been used according to the manufacturer's instruction.

Animals

Athymic nu/nu BALB/c female mice were purchased from the Jackson Laboratory (Bar Harbor, ME).

Tumor cell growth in mice

Human breast tumors were generated by injecting athymic nude mice with 5×10^5 MDA-MB-231 cells subcutaneously. After 10–14 days, the mice were checked for tumor development and only mice with palpable tumors were subsequently injected intravenously with biodegradable gelatin microspheres containing 100 μ g of either anti-fragment mAbs or irrelevant antihuman HLA IgG₁ mAb. Mice

were sacrificed at various intervals and tumor size was evaluated by weighing of the tumors. Eight weeks after the initial injection of breast cancer cells (left), mice from the anti-AA 27-44 mAb-treated group were secondarily challenged with prostate cancer cells. Again, the mice were divided into several groups and treated by mAb. The original groups consisted of 10 mice per group, the second part of the experiment used three mice per group. Mice were sacrificed at various time intervals and tumor size was evaluated by weight. Biodegradable gelatin microspheres were prepared as described by Golumbek et al.⁵²

In immunization experiments, mice were immunized with 25 µg of activation peptide in complete Freund adjuvant (four immunizations in 6-week interval). After checking the level of antiactivation peptide antibodies by ELISA (results not shown), mice were injected with human tumor cells as described above. The mice were sacrificed at various intervals as described.

Results

Because only the enzymatically inactive pCD is secreted from cancer cells, we assumed possible relationship between procathepsin and the development of breast cancer. These studies demonstrated that pCD had an autocrine growth factor activity on breast cancer cells that was mediated by a specific, so-far-unknown receptor, which seems to recognize structure within the activation peptide of pCD (27-44 AA sequence⁴⁷ and probably even 36-44 AA).

pCD was tested by cultivation of tumor cell lines from different tissue origins in serum-free medium. Cell growth was monitored by an MTT assay at 570 nm (Fig 1). pCD only had mitogenic activity for mammary tumor cell lines. The growth of other cell lines was not sustained with pCD in serum-free medium. Additional tumors not responding to pCD include U937, HeLa, and HepG2 lines.⁵³ We have also compared the effects of pCD to the effects of insulin-like growth factor II (IGF II), a known mitogen for many human cell lines. The results showed that the growth potentiation

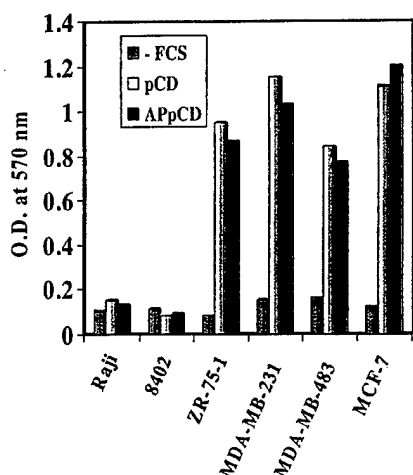


Figure 1 Growth of human cell lines in serum-free medium containing either 40 ng/mL pCD or 50 ng/mL activation peptide (APpCD).

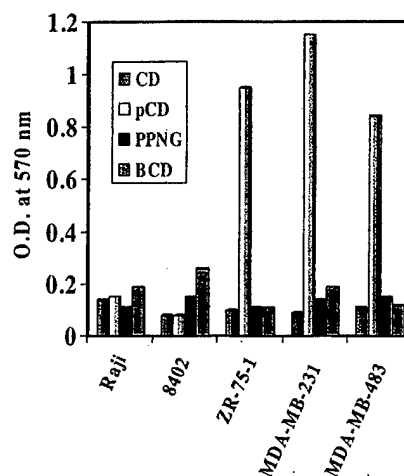


Figure 2 Growth of human cell lines in serum-free medium containing 2 nM human cathepsin D (CD), 2 nM pCD, 20 nM pepsinogen (PPNG), or 2 nM bovine cathepsin D (BCD).

effects of IGF II on breast cancer cells are identical to those of pCD, but IGF II stimulated the proliferation of all types of cancer cells we tested.⁵³ Additional experiments were focused on potentially comparing the effects of pCD to the mature human and bovine CD or pepsinogen. No proliferative activity was observed for any of these control molecules (Fig 2).

Experiments were then performed to determine whether the mitogenic activity could be mediated by the M6P-R or a yet different receptor. First, high concentrations of M6P known to inhibit the M6P-R-mediated interaction and internalization of pCD⁵⁴ were used as a specific inhibitor of the M6P-R. The mitogenic function of pCD was not blocked by high concentrations of M6P.⁵⁵ Figure 3 further demonstrates that the growth factor activity of pCD was not blocked by either of two types of anti-M6P-R antibodies (either calcium-dependent 46-kDa or calcium-independent 300-kDa receptor) nor by competing soluble M6P-R. Finally, there was very little loss of activity when deglycosylated pCD was used, indicating that pCD has growth factor

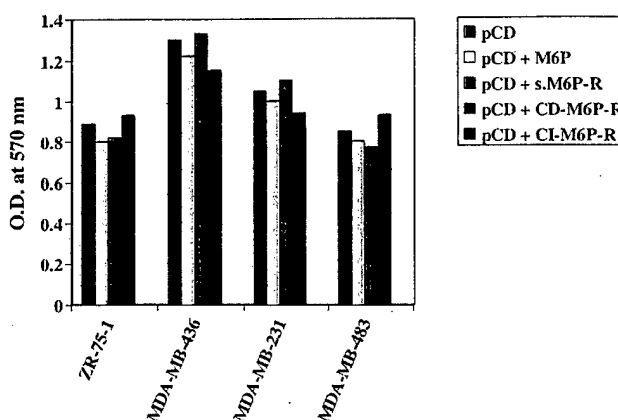


Figure 3 Effect of various potential inhibitors on pCD-mediated growth of human cell lines. The concentrations were used as follows: 40 ng/mL pCD; 10 mM M6P; 1 µg/mL soluble M6P receptor (s.M6P-R); 1 µg/mL anti-M6P-R antibodies.

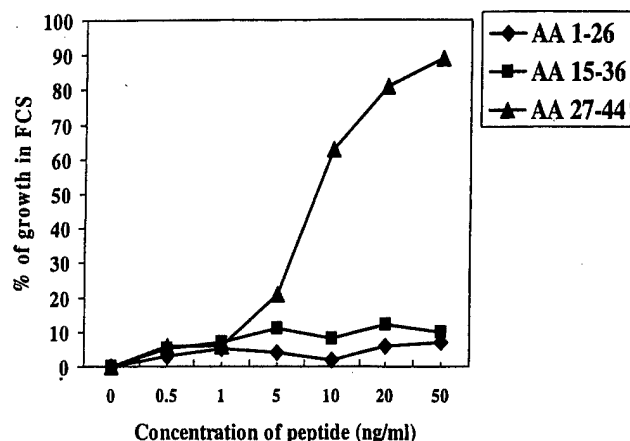


Figure 4 Effect of various concentrations of three synthetic peptides representing fragments of activation peptide on the growth of human breast cancer cell line ZR-75-1 in serum-free medium.

activity that is not mediated by an interaction of M6P with the M6P-R.

At this stage, all the experimental evidence indicated that a pivotal role in the mitogenic activity is included in the activation peptide of the pCD. The mitogenic function of pCD was further tested by using activation peptide alone or various synthetic peptides based on modeled 3D structure of pCD.⁴⁸ All these results suggested that the mitogenic function of activation peptide was mediated through a specific receptor expressed on all eight human breast cancer cell lines examined. Scatchard plot analysis revealed that the number of binding sites was 1.83×10^4 per cell, with a K_d of 0.61 nM.⁵⁶ Next we prepared 10 synthetic peptides representing several parts of the activation peptide and compared their mitogenic activity. Our data indicated that the binding site of APpCD is located somewhere between amino acid positions 27 and 44.^{47,56} All peptides representing first 1–27 AA of the APpCD peptide were not effective (Fig 4); AA 27–44 peptide was effective at similar doses to those found with the APpCD. Subsequent experiments demonstrated that the active part of the APpCD can be located to the smaller peptide AA 36–44 (data not shown). This information should be very useful as we screen for potential pCD receptors. In addition, significant inhibition of breast cancer growth *in vitro* was achieved by anti-AA 27–44 fragment antibodies.⁴⁷ After determining that the growth-stimulating effects of pCD and its activation peptide may be mediated through a new, previously unknown receptor, we investigated if the activation peptide is similar to other known proteins. When searching for the receptor structure in PIR and SWISSPROT databases, using the Fasta program against all known human proteins, only significant similarities to other human aspartic proteinases were found. No additional similarity was considered to be significant.

Subsequent experiments demonstrated that prostate cancer cells PC3, DU145, and LNCaP^{38,57} and ovarian cancer cells UL-1 and SKOV-3⁵⁸ are similarly sensitive to the pCD treatment. Furthermore, this mitogenic activity was blocked by antibodies to APpCD. Finally, there was no mitogenic

activity detected when a peptide of the same size but with a scrambled sequence was used (data not shown).

Next, the growth-promoting activity of pCD was demonstrated in an *in vivo* animal model in which anti-pCD antibodies were shown to reverse breast cancer development. Human breast tumors were generated by injecting athymic nude mice with 5×10^6 MDA-MB-231 cells (which lack the estrogen receptor) directly into mammary fat pads. After 2 weeks, the mice were checked for tumor development. Mice with palpable breast tumors were then injected intravenously with biodegradable gelatin microspheres containing 100 μ g of either pCD, anti-pCD mAb, anti-fragment 1–27 mAb, anti-fragment 36–44 mAb, or irrelevant anti-HLA mAb (control Ab) or the same isotype. Mice were sacrificed at various time intervals and tumor size was evaluated by weight. Mice treated with either anti-pCD or anti-fragment 36–44 antibodies showed greatly reduced tumor growth.⁴⁷ pCD alone increased the tumor growth only slightly over the control group, no doubt due to a short *in vivo* lifespan of pCD. Subsequent study measured the survival rate of mice treated with either anti-fragment 27–44 antibodies or antihuman factor I mAb after challenge with MDA-MB-231 cells. Again, antibodies were coupled with gelatin microspheres. Ten days after subcutaneous injection of tumor cells, the mice were given the antibody. This treatment was repeated on day 45 after original challenge with tumor cells (Fig 5).

To examine the feasibility of our proposal even further, we tested the effects of anti-fragment antibody treatment on mice challenged with secondary cancer (Fig 6). The treatment started 10 days after first tumor cells injection. Eight weeks after the initial injection of breast cancer cells (left), mice from the treated group were secondarily challenged with prostate cancer cells. Again, the mice were divided into several groups and treated by mAbs (right). The original groups consists of 10 mice per group; the second part of the experiment used three mice per group. A control mAb (anti-HLA) of the same isotype or mAb against AA 1–26 fragment had no effect. The results of all animal experiments showed the feasibility of developing agents that block the mitogenic activity of pCD in human breast tumors. Similarly, we have used the complete activation peptide

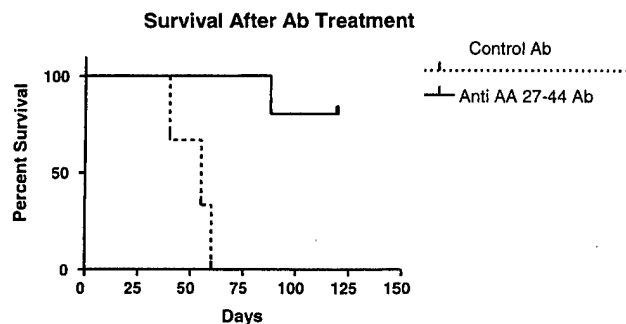


Figure 5 Survival of groups of six mice treated with anti-AA 27–44 mAb or antihuman factor I mAb after challenge with the MDA-MB-231 cells. Ten days after subcutaneous injection with breast cancer cells, the mice were given treatment by intraperitoneal injection. The treatment was repeated on day 45 after original challenge.

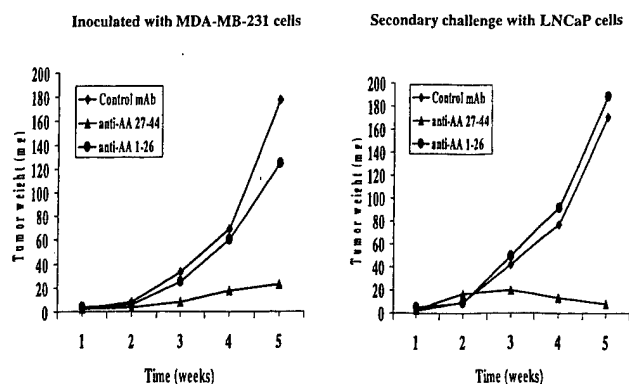


Figure 6 Inhibition of cancer growth in athymic mice by treatment with anti-fragment mAb. A control mAb (anti-HLA) of the same isotype or mAb against AA 1–26 fragment had no effect. The treatment started 10 days after tumor cells injection. Eight weeks after the initial injection of breast cancer cells (left), mice from the treated group were secondarily challenged with prostate cancer cells. Again, the mice were divided into several groups and treated by mAb (right). The original groups consist of 10 mice per group; the second part of the experiment used three mice per group.

for immunization of athymic mice prior the tumor cell implantation (see Fig 7). The results of immunized and control animals were analyzed in the same way as in previous experiments.

In the final experiment, we focused our attention on possible inhibition of pCD synthesis using specifically designed ribozymes. One of the problems with the design of ribozymes is the identification of potential cleavage sites on the target RNA because RNA folds into complex secondary structures, which interfere with binding of the ribozyme. Efficient ribozyme cleavage sites on long substrates cannot be easily predicted. We used MFOLD Program version 3.1. for prediction of the most stable secondary structure of pCD mRNA using energy minimiza-

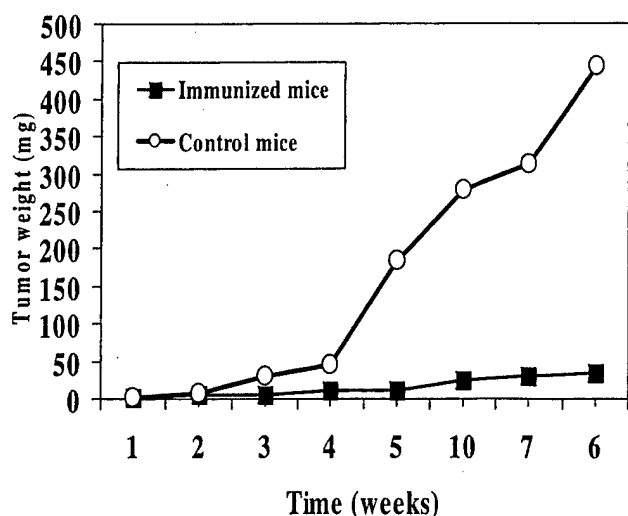


Figure 7 Athymic nude mice were immunized by activation peptide as described in *Materials and methods*. Injection with cancer cells and subsequent evaluation of mice were identical to that in Figure 6. Day of cancer cells application is considered as day 0.

tion method.⁵⁹ Ribozyme target sites that are part of, or in close proximity to, open loop regions and that contain NUH sequence motif (N is any nucleotide and H is C, U, or A) were selected. The preference was given for GUH sites (especially GUC sites) because the highest ribozyme cleavage efficiency has been shown for these sites.⁶⁰ Seven hammerhead ribozymes were designed. These were tested using the same program to determine whether the selected sequences are able to fold into a typical hammerhead ribozyme structure. Finally, we tested the selected ribozyme sequences for specificity against other known human sequences using the BLAST program.⁶¹

Designed ribozyme sequences:

RZ385: 5'-GUGCUGCUgAUGAGUCCGUGAGGAC-GAAACUUGUCGUGU-3'
RZ763: 5'-UUGUAACUgAUGAGUCCGUGAGGAC-GAAACUUGGAGUCUG-3'
RZ774: 5'-ACAGAGCUgAUGAGUCCGUGAGGAC-GAAACCUUGUAAUAC-3'
RZ644: 5'-GUCGAACUgAUGAGUCCGUGAGGAC-GAAACGGGCAGCAC-3'
RZ1127: 5'-UGGCGGCUgAUGAGUCCGUGAGGAC-GAAAUGUCCAUGC-3'
RZ94: 5'-CGGAUGCUgAUGAGUCCGUGAGGAC-GAAACGUGAACUUG-3'
RZ1254: 5'-CGCGCGCUgAUGAGUCCGUGAGGAC-GAAACGCCUUGGGAA-3'.

Ribozymes were numbered according to the position of their cleavage site in pCD mRNA sequence. Ribozyme RZ94 was designed to target pCD mRNA in AP sequence. Sequences of antisense and control sense oligodeoxyribonucleotides corresponding to ribozyme target sequences identified by MFOLD program were as follows:

antisense RZ385: 5'-GTGCTGGACTTGTCGCTGT-3'
antisense RZ763: 5'-TTGTAATACTTGGAGTCTG-3'
antisense RZ774: 5'-ACAGAGAACCCTTGTAAATAC-3'
antisense RZ644: 5'-GTCGAAGACGGGCAGCAC-3'
antisense RZ1127: 5'-TGGCGGGATGTCCATGC-3'
antisense RZ94: 5'-CGGATGGACGTGAACTTG-3'
antisense RZ1254: 5'-CGCGCGGACGCCUUGGGAA-3'
sense control: 5'-ACAGCGACAAGTCCAGCAC-3'.

For evaluation of the invasion across Matrigel layer, we used a commercial kit. The results summarized in Table 1

Table 1 Invasiveness through Matrigel membrane

Cell line	Transfected with plasmid coding			
	Original line	Control	pCD	RZ644 ribozyme
HBL-100	–	–	++	–
ZR-75-1	+	–	+++	–

The kit was used according to manufacturer's instruction. Each experiment was done in triplicate.

clearly show that transfection with pCD cDNA increased the invasion in both control (previously negative cell line) and in breast cancer cell line ZR-75-1. In addition, transfection of these lines with specific ribozyme RZ644 resulted in inhibition of invasion. Transfection with control sense resulted in no changes (data not shown).

Discussion

Secreted pCD was demonstrated by immunofluorescence in tissue section, and by ELISA in tissue culture supernatants, carcinoma cytosols, saliva,⁶² or nipple aspirates of both normal and carcinoma tissues (for review, see Ref. [46]). Moreover, several clinical studies suggested a potential role for this molecule in metastasis because its concentration in primary tumors correlated with an increased incidence of tumor metastases.^{14,15,63} In nude mice, it was shown that rat tumor cells were converted from low to high metastatic potential by transfection with the cDNA for human CD.^{64,65} indicating the role of pCD in metastases. The major question remains: What mechanism is employed in the cancer-promoting activity of pCD?

In the initial experiments, Rochefort and Capony⁶⁶ hypothesized that hypersecretion of pCD caused and/or regulated by estrogens is followed by maturation at the unknown site, which suggests the role of mature CD instead of zymogen pCD. This possibility was further increased by experiments employing rat cancer cell line transfected with human CD cDNA. Clones transfected with regular CD cDNA developed larger metastases than clones transfected with vectors where KDEL signal retained pCD in the endoplasmic reticulum.⁶⁷ However, the addition of the KDEL signal inhibited maturation of pCD, not the secretion of it. Thus, the conclusion of these experiments remains controversial. Later reviews discussed the possible dual role of (pro)cathepsin D in cancer development — acting as a protease after its activation and/or as a ligand on membrane receptor before activation.^{10,12}

Secreted pCD might be activated by acidic extracellular conditions and subsequently degrade growth inhibitors and extracellular matrix, or liberate some growth factors. The possibility of the extracellular matrix degradation seems to be supported by findings of Briozzo et al,⁶⁸ but their experiments showing liberation of FGF₂ from extracellular matrix are rather difficult to interpret. An important observation, using a mutated CD with abolished proteolytic activity, showed stimulation of cancer cell proliferation both *in vivo* and *in vitro*.⁶⁹ These experiments clearly indicated that proteolytic activity is not involved in the “cancer-promoting” or mitogenic function of pCD in certain cancer tissues.

When the proteolytic activity is ruled out as the mechanism of mitogenicity, the second possibility is that pCD is (as a whole molecule) directly involved in cancer cell growth and/or differentiation. To test this, it was necessary to demonstrate specific binding of pCD. Hence, at least two types of receptors are known to interact with pCD. The first type is represented by two different M6P/IGF II receptors (for review, see Ref. [10]). These two receptors involved in

the lysosomal targeting of pCD are expressed both intracellularly and on the outer cell membrane. Despite the fact that intracellular targeting of pCD through M6P is well established,^{70–72} studies indicating an alternative targeting of pCD, both intra- and extracellularly, are common^{53,73–77} and represent a second known interaction of pCD with a receptor. This independence on established M6P-R binding is even more pronounced in breast cancer cells. Rochefort et al analyzed pCD uptake in two human breast cancer cell lines and showed that the internalization is independent on M6P. Additional experiments showed the presence of saturable, M6P-independent binding sites.⁷⁸ The proofs of the new pathway of pCD interaction with cell membrane were further strengthened by the finding that the binding of FITC-labeled pCD to cancer cells can be inhibited by unlabeled procathepsin but only marginally by either M6P, anti-M6P-R antibodies, or by soluble M6P-R.^{56,79} In addition, the biological activities of pCD cannot be blocked by anti-M6P-R antibodies.

After establishing the interaction of pCD with a putative cell surface receptor, we focused on experiments testing the hypothesis that structure responsible for mitogenicity of pCD is contained within the APpCD. An important condition for such a function of the APpCD — the localization of the propeptide on a surface of the pCD molecule — is fulfilled.⁴⁸

The effects of various concentrations of either pCD or APpCD on proliferation of various breast cancer cells lines were described.^{53,56} When growth factor activity of these molecules was evaluated, irrelevant synthetic peptides with similar purity and molecular weight (peptide representing the intracellular part of CR3 receptor and pig pepsinogen A) were used.⁴⁷ The optimal concentration of APpCD was found to be around 50 ng/mL, a dose slightly higher than required for the parent pCD molecule. The differences in effective molar concentration between APpCD and pCD can be explained on the base of the 3D structure of CD and a model of 3D structure of pCD.⁴⁸ It is clear that the conformation of the activation peptide in the pCD is stabilized by numerous noncovalent bonds as well as a covalent attachment of APpCD to the N-terminus of a mature molecule and, therefore, the activity of the peptide is less effective.

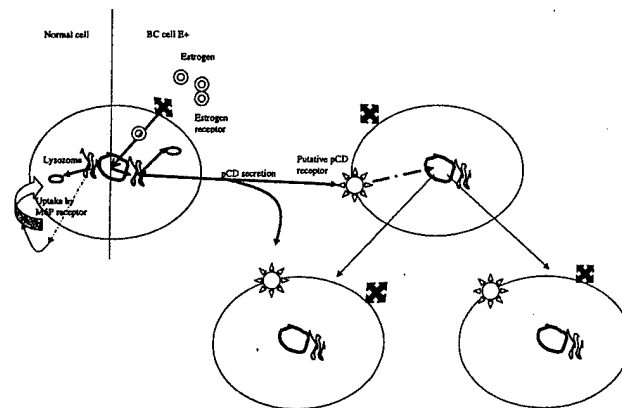


Figure 8 Hypothetical mechanism of pCD involvement in cancer development and progression.

The question of whether the pCD found in cytosols of human breast tumors originates from disintegrating cells or from active secretion has not been adequately addressed. Using technique originally developed for staining intracellular cytokines, we showed not only the presence of pCD inside tumor cells, but also a significant increase of pCD staining when cells were incubated with the protein transport inhibitor brefeldin A.⁵⁶ Identical results were obtained when monensin, instead of brefeldin A, was used. The specificity of this secretion was shown by double staining with anti-MUCIN-1 mAb, which specifically stained breast cancer cells. Eight different tumors were tested with identical results. These results clearly indicate that pCD is actively secreted by breast cancer cells.

The role of pCD has recently been demonstrated by using a retroviral approach. Overexpression of pCD increased colony formation in NIH3T3 cells and progression of prostate cancer.⁸⁰ Thus, studies in this and other laboratories appear to implicate pCD in growth and/or metastasis of breast cancer. However, few tumor cell lines have been investigated in this regard and many important questions remain with respect to mechanisms. For example, no quantitative information is available to assess a potential correlation between levels of pCD expression and aggressive tumor behavior. Carefully blinded studies will assess transfectants for growth in serum-free medium and invasion in culture, as well as growth and metastasis *in vivo*. In contrast to our results, Liaudet-Coopman et al⁶⁹ have not been able, under their experimental conditions, to confirm the role of the APpCD in mitogenesis. The experiments with mutated pCD have also concluded that the mitogenic function was independent on M6P, suggesting the possibility of an involvement of yet additional surface structure on pCD and additional unknown receptor. Nevertheless, Altschul et al⁶¹ have been able to show similar behavior of the APpCD in the case of endometrial tumor-derived cell lines.

Based on the results shown in numerous papers,^{47,55–57,79} as well as the data of Chinni et al,⁴¹ we suggest the following mechanism (Fig 8): In normal cells, pCD is targeted to lysosomes using an M6P tag and, parallel to that, by a yet unknown ligand–receptor interaction. The small part of pCD that eludes this targeting and might be secreted is recaptured through M6P receptors expressed on the cell surface. In breast cancer cells, overexpression of pCD induced by estrogens occurs, with subsequent secretion of the major part of the “extra” pCD. Due to massive overexpression, saturation of M6P receptors occurs and the recapturing is abolished. The remaining extracellular pCD interacts with additional cancer cells in close vicinity of the secreting cells through putative activation peptide receptor. The interaction is performed by APpCD of pCD with an unknown receptor. This interaction results in higher proliferation of cancer cells.

When antibodies specifically recognizing pCD have been used, two groups reported findings of elevated concentration of pCD in serum of metastatic breast cancer patients,^{81,82} suggesting that, at least in final stages of cancer, pCD is released into the bloodstream. It is important that the

activation peptide sequence is recognized by the immune system as a non-self-structure and triggers the production of specific antiactivation peptide antibodies.^{41,83}

Preliminary experiments using specifically designed ribozymes demonstrated not only that we can influence the synthesis of pCD in cancer cells, but also their characteristics such as invasion across Matrigel layers (Table 1).

Finally, our hypothesis accenting the role of the APpCD in tumor cell growth explains all the experimental data we have gathered. Such hypothesis also offers interesting possibilities for therapeutic use. Direct experiments using antibodies blocking the putative interaction of AP with the receptor in animal model are described in this paper. Blocking of this interaction has caused fundamental changes in growth of carcinoma in experimental animals. The last set of our experiments, using active immunization of mice by the activation peptide with subsequent transfer of the carcinoma, shows possible consequences of this hypothesis. Again, there is considerably stronger suppression of carcinoma growth in the animals that were immunized compared to those without pretreatment.

Acknowledgments

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Cytokines Affect Procathepsin D-stimulated Proliferation of Breast Cancer Cells

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Abstract. Procathepsin D is over secreted by certain human cancer cells. This enzymatically inactive precursor has been established as playing an important role in the development of several types of cancer. Due to their pleiotropical effects, numerous cytokines have also been recognized as important immunotherapeutic agents. In this study, we focused on the role of IL-4, IL-10 and IL-13 on procathepsin D-stimulated proliferation of breast cancer cells. Our results clearly showed that only ER+ breast cancer cells responded to the presence of cytokines by proliferation; ER- cells were resistant to the addition of cytokines. Since addition of anti-procathepsin D antibodies blocked the growth potentiation, we concluded that addition of these cytokines resulted in stimulation of synthesis and/or release of procathepsin D. This conclusion was further supported by findings of procathepsin D in culture supernatants of cells incubated with cytokines.

Numerous clinical studies has shown that cathepsin and procathepsin D levels in primary breast cancer cytosol serve as an independent prognostic factor (for review see 1,2). In addition, both procathepsin D and antibodies against individual fragments of procathepsin D activation peptide have been found in sera of breast and ovarian cancer patients (3-5).

The intracellular processing and maturation has been described in full detail (6, for review see 3). Similarly, cell trafficking of procathepsin D has been studied for a long time. Cathepsin D, in the form of proenzyme, is normally targeted to lysosomal compartments and resides inside cells. In certain tissues the synthesis of cathepsin D is controlled by steroid hormones. Progesterone and its derivatives increase the rate of uterine cathepsin D synthesis (7) and in breast cancer cell lines, cathepsin D expression is regulated by

estrogens (8) that interact at the promoter level (9). In ER+ cell lines, procathepsin D is secreted only after estrogen stimulation. A short proximal promoter fragment from -365 to -122 is required for this induction. Rochefort's group characterized a nonconsensus estrogen-responsive element (ERE) with two differences in the distal half of its palindrome. This site is necessary but not sufficient to mediate an estrogen response and required cooperation with the homologous E1 element. The authors concluded that estradiol induction of CD is mediated by interaction of the estrogen receptor with a nonconsensus ERE that requires synergy with other elements located upstream of this central ERE (10). The structure and function of the proximal 5' upstream region of the human CD gene was determined. The promoter has a compound structure with features of both housekeeping genes and regulated genes. The transcription was shown to be initiated at five major transcription sites spanning 52 base pairs. Estradiol was found to increase 6- to 10-fold the level of RNA initiated at the transcription site I located about 28 base pairs downstream from the TATA box. Site-directed mutagenesis indicated that the TATA box is essential for initiation of CD gene transcription. Depending on conditions, the CD gene behaves either as a housekeeping gene with multiple start sites or as a hormone-regulated gene that can be controlled from its TATA box (9). Despite all these studies, little is known about the possible effects of bioactive substances on procathepsin D secretion.

Procathepsin D is, via its over expression and subsequent secretion, involved in several types of cancer, mostly breast and ovarian cancer (6, 11-15). Cell proliferation experiments demonstrated mitogenic properties of procathepsin D (16), while subsequent analysis of the interaction of procathepsin D with the cancer cell membrane showed first that the active structure of procathepsin D responsible for the mitogenic effect is located in the 44 amino acid activation peptide (AP) (13, 17). A follow-up of these studies helped localize the active moiety even further up to a nine amino acid stretch AA 36-44 (18).

Human carcinomas are frequently infiltrated by inflammatory cells secreting several cytokines; some cytokines have been identified as important regulators of cancer cells.

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For example, the level of cytokine production by cancer cells is different compared to normal tissue (19), which is often considered to be one of the reasons for escaping immunosurveillance and control. In addition, many cytokines have been shown to play an important role in cancer development. After numerous studies of the effects of IL-2 and IL-12, the focus shifted towards IL-4, as receptors for IL-4 are present on a large number of tissue specimens isolated from cancer tissue (20). Interleukins -4 and -13 strongly inhibited estrogen-induced proliferation of breast cancer cells while they stimulated the GCDFFP-15 expression in these cells (21, 22). Similar effects of IL-4 and IL-13 might reflect the fact that they bind to the receptor that shares a common gamma chain. Levels of IL-6 and IL-8 were even suggested as predictive indicators of the therapeutic response in breast cancer patients (23). Due to their pleiotropical effects, numerous cytokines have also been recognized as important immunotherapeutic agents (24). However, despite numerous studies, these cytokines have antiproliferative effects on the growth of only some cancer cell types and the mechanisms remain to be elucidated.

Previous studies have demonstrated the regulatory effects of IL-4, IL-10 and IL-13 on lysosomal enzyme secretion by lamina propria mononuclear cells and peripheral blood monocytes isolated from patients with inflammatory bowel disease (25). In these patients, lipopolysaccharide-stimulated secretion of both the precursor and the mature form of the cathepsin D in monocytes was shown to be inhibited by all three tested cytokines. The precursor form represents a fraction of newly-synthesized lysosomal enzymes that escaped segregation to the lysosomes. In addition, cytokines have been shown to stimulate intracellular levels of cathepsin HmRNA (26). Based on these studies, we decided to test these three cytokines for their potential effects of synthesis and secretion of procathepsin D and subsequently proliferation of breast cancer cells.

Materials and Methods

RPMI 1640 medium, Iscove's modified Dulbecco's medium, HEPES, MTT, antibiotics, Limulus lysate test E-TOXATE and transferrin were obtained from Sigma Chemical Co. (St. Louis, MO USA), fetal calf serum (FBS) was from Hyclone Laboratories (Logan, UT, USA), while human recombinant IL-4, IL-10 and IL-13 were purchased from Biosource International (Camarillo, CA, USA).

Monoclonal antibodies against activation peptide have been described previously (13). IgG was isolated from ascites fluid by 50% ammonium sulfate precipitation followed by Mono-Q anion exchange chromatography. Monoclonal anti-cathepsin D antibodies were purchased from Calbiochem-Novabiochem Corporation (La Jolla, CA, USA). Control MOPC-21 IgG antibody was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Monoclonal anti human IL-4, IL-10 and IL-13 antibodies were purchased from Endogen (Woburn, MA, USA).

The 44-amino-acid-long peptide corresponding to the activation peptide of pCD was synthesized in the Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague, Czech Republic. The purity of the AP was controlled using the following methods: HPLC, amino acid analysis and by mass spectrometry. The

HPLC method showed the purity to be more than 95%. The amino acid analysis confirmed the amino acid composition while mass spectrometry results were in accordance with the proposed molecular structure. In addition to the techniques mentioned above, the purity of the peptide also controlled by was terminal sequencing using an automated system where the first 9 -terminal amino acids were in agreement with the designed structure.

The human breast cancer cell lines MCF-7, MX-2 and BT-20 were obtained from the American Tissue Culture Collection (ATCC, Rockville, MD, USA). Human breast cancer cell lines ZR-75-1, MDA-MB-483 and MDA-MB-231 were obtained from Dr. R. Ceriani of the John Muir Cancer and Aging Research Institute, Walnut Creek, CA, USA. The cancer celllines were grown in RPMI 1640 medium with HEPES buffer supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin in plastic disposable tissue culture flasks at 37°C in a 5% CO₂/95% air incubator.

For growth experiments, the cells were first incubated for 2 days in 0.1% FCS. The cells were harvested by centrifuging and washed six times in Iscove's modified Dulbecco's medium with HEPES buffer supplemented with glutamine, antibiotics and 10 µg/ml of human transferrin (17). The cells were seeded in 96-well tissue culture plates at a density of 5x10⁴ cells/ml (150 µl/well) in the presence or absence of different concentrations of purified activation peptide or various substances tested in triplicate wells. After six days in culture, the proliferation was evaluated using an MTT assay (17). In all cell culture experiments, the cells were seeded in triplicates. We repeated our experiments using both charcoal-treated FBS and medium without phenol red with identical results.

For analytical experiments, the cells were incubated in 96-well tissue culture plates at a density of 5x10⁴ cells/mL (150 µl/well) in the presence or absence of different concentrations of cytokines in triplicate wells. After 5 days of incubation, the supernatants were collected and stored at -80°C.

The supernatant samples were analyzed using SDS electrophoresis according to Laemmli (27) under reducing conditions. The same amounts of each sample were diluted in a ratio 1:1 with reducing sample buffer. The samples were analyzed by 5% acryl amide stacking gel and 12% acryl amide running gel (buffers and solutions equipment as well as secondary antibodies were purchased from Sigma).

Separated proteins were then transferred on the PVDF membrane using Western blotting. After transfer the membrane was first soaked in 3% skimmed milk and three times washed with PBS/0.03% TWEEN 20. The primary antibodies (polyclonal rabbit antibodies raised against the propetide) were then applied overnight. After four washes with PBS/0.03%, TWEEN 20 secondary antibodies (anti rabbit IgG conjugated with peroxidase, dilution 1:500 in PBS) were applied for 1 hour at room temperature. The membrane was then washed 4x with PBS/0.03% TWEEN 20 and developed using chemiluminescence. The solutions used for this development were as follows: A) 1 ml of 1M Tris-HCl, pH 8.5, 100µl 250 mM Luminol in DMSO, 44µl of 90 mM p-coumaric acid in DMSO mixture adjusted to 10 ml by deionized water; B) 6 µl of 30% hydrogen peroxide, 1 ml of 1M Tris. HCl pH 8.5, adjusted with deionized water to 10 ml. These two mixtures were combined and PVDF membrane was soaked in the solution for 1 minute, then covered with transparent wrap and applied on X-ray film before the film was finally developed.

Results

Figure 1 shows the effect of various concentrations of human recombinant interleukins IL-4 on the proliferation of six different human breast cancer cell lines cultivated in serum-free conditions. Similarly, Figures 2 and 3 demonstrate the effects of addition of either IL-10 (Figure 2) or IL-13 (Figure 3) on breast cancer cell growth. The breast cancer cells lines

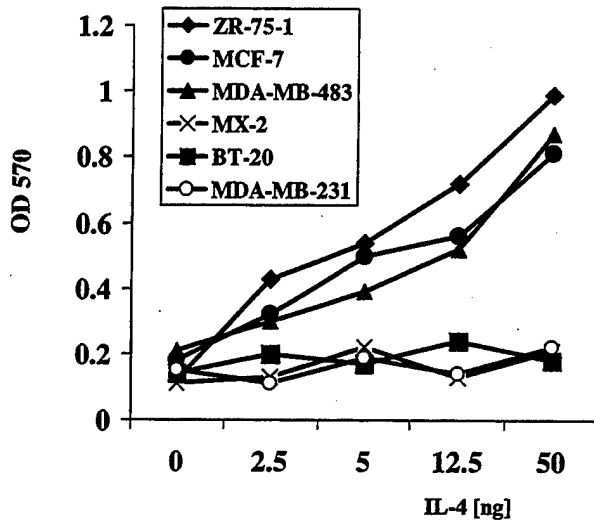


Figure 1. Growth of human breast cancer cells in serum-free medium containing different doses of human recombinant IL-4. After six days culture, the proliferation was evaluated using an MTT assay. The results represent the mean of three independent experiments.

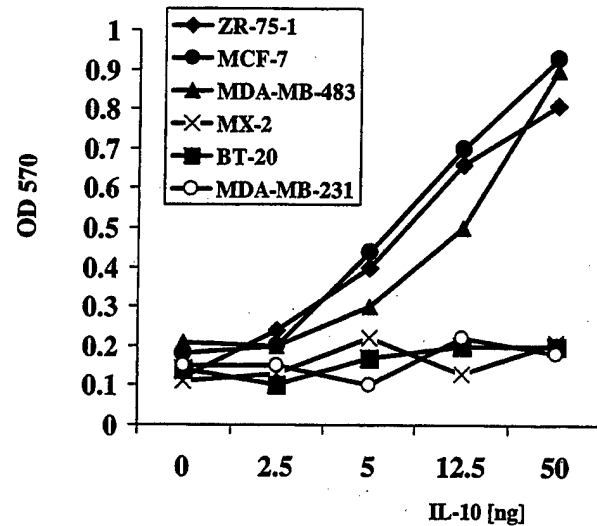


Figure 2. Growth of human breast cancer cells in serum-free medium containing different doses of human recombinant IL-10. After six days in culture, the proliferation was evaluated using an MTT assay. The results represent the mean of three independent experiments.

used in our study can be divided into two groups: cell lines ZR-75-1, MCF-7 and MDA-MB-483 are estrogen receptor-positive; the rest of the tested cell lines were estrogen receptor - negative. The results summarized in Figures 1 to 3 clearly showed that only ER+ responded to the presence of all three tested cytokines by proliferation. As the cells were cultivated in serum-free and estrogen-free conditions, the proliferation was potentiated only by the added cytokines.

As the results suggested that these three interleukins might affect breast cancer cells with the same effects as estrogens, we tried to ensure that the observed effects were in reality caused by added interleukins and not by either procathepsin or estrogen already present in the culture media. All media were tested for endotoxin contamination and shown to contain ≤ 0.1 ng/ml LPS using the Limulus lysate test. Steroid-deprived cells were used in all experiments. The levels of procathepsin D in serum in the concentration used were below detection levels. Similarly, the levels of estrogens in serum and in tissue culture were ≤ 1 pg/ml (data not shown).

These experiments demonstrated the significant stimulation of proliferation of ER+ breast cancer cells. To further investigate the hypothesis that this growth potentiation has a similar pattern to the potentiation by estrogen, which is mediated by changes in synthesis and/or secretion of procathepsin D, we measured the interleukin-mediated proliferation of cell line ZR-75-1 after addition of various concentrations of anti-procathepsin D antibody. The addition of specific anticytokine antibodies served as positive control, anti-cathepsin D or control MOPC-21 antibodies of the same

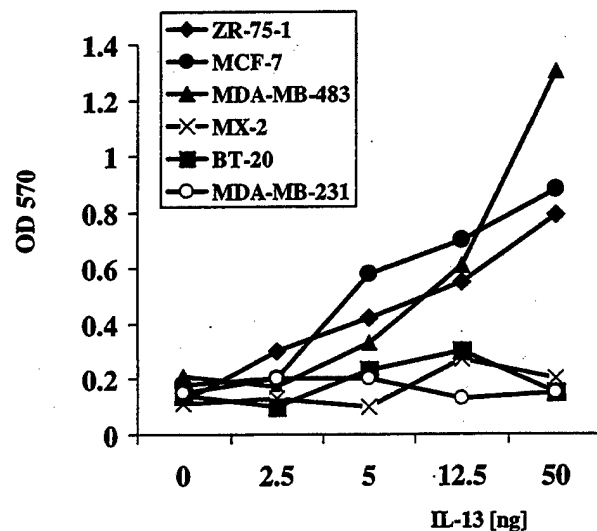


Figure 3. Growth of human breast cancer cells in serum-free medium containing different doses of human recombinant IL-13. After six days in culture, the proliferation was evaluated using an MTT assay. The results represent the mean of three independent experiments.

isotype served as negative controls. The results of these experiments are summarized in Figures 4 to 6. When we simultaneously added two different cytokines, they showed only a slight and nonsignificant additive stimulation of breast cancer (data not shown).

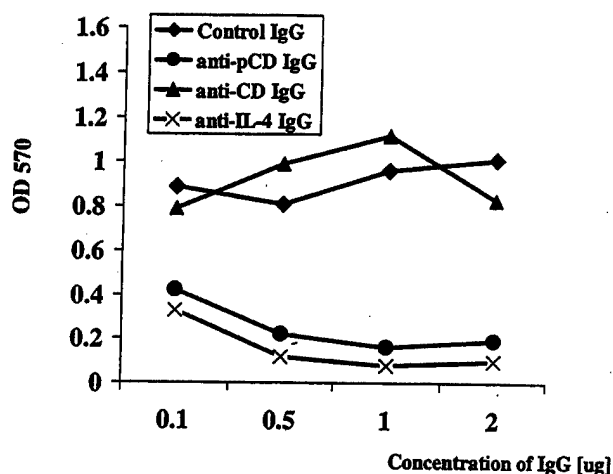


Figure 4. Inhibition of the rIL-4 induced growth of human breast cancer cell line ZR-75-1. The cells were simultaneously incubated with 10 ng of rIL-4 and various concentrations of either anti-procathepsin D IgG, anti-cathepsin D IgG, anti-IL-4 IgG or control MOPC-21 IgG. The results represent the mean of three independent experiments.

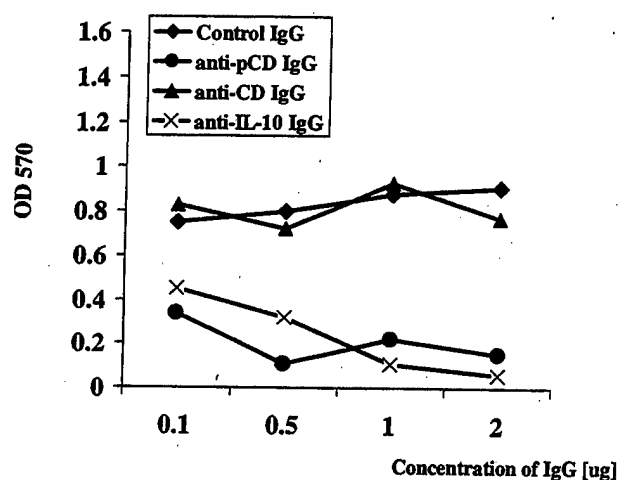


Figure 5. Inhibition of the rIL-10-induced growth of human breast cancer cell line ZR-75-1. The cells were simultaneously incubated with 10 ng of rIL-10 and various concentrations of either anti-procathepsin D IgG, anti-cathepsin D IgG, anti-IL-10 IgG or control MOPC-21 IgG. The results represent the mean of three independent experiments.

In earlier work we have shown the response of the above-mentioned cell lines to the addition of estrogen in the respect of procathepsin D secretion (16). Experiments described in this paper suggested that the effects of the tested interleukins IL-4, IL-10 and IL-13 are very similar to the effects of estrogen in stimulation of procathepsin D secretion from ER+ breast cancer cell lines. To gain direct evidence of the fact that the interleukins stimulate the secretion of procathepsin D, we grew the tested cell lines in serum-free medium in the presence or absence of the tested interleukins. After two days of incubation, the medium was harvested and stored. Later, the medium was concentrated and applied on SDS electrophoresis and subsequently blotted and visualized by chemiluminescence. The results seen in Figure 7 clearly show that incubation of the ZR-75-1, MCF-7 and MDA-MB-483 cells with each of our three interleukins resulted in the secretion of pCD into the medium, while MX-2, BT-20 and MDA-MB-231 cells did not respond by pCD secretion. Preliminary results show that levels of pCD secreted by ER+ cells after potentiation with estrogens and with interleukins is comparable.

Discussion

In this study, we focused on the elucidation of possible effects of three different cytokines on procathepsin D-derived stimulation of breast cancer cell proliferation. The three cytokines increased cellular growth and, in parallel, we found increased secretion of procathepsin D from ER+ cells. Procathepsin D has been found to have a significant growth

factor activity via an autocrine fashion (13, 17). Despite numerous studies, the effects of estrogens on procathepsin D synthesis and release in breast cancer cells is still not clear. On one hand, in ER+ cell lines, procathepsin D is secreted only after estrogen stimulation and this stimulation of pCD secretion can be specifically inhibited by specific estrogen inhibitor 2,3,7,8-tetrachlorodibenzo-p-dioxin (28). On the other hand, procathepsin D is secreted constitutively in ER-cancer cell lines (29). The connection between estrogen levels and procathepsin D secretion was further demonstrated in experiments showing that the estrogen-induced *in vitro* proliferation of cancer cells can be inhibited by anti-procathepsin D antibodies (18). Further experiments revealed that both estrogen and activation peptide stimulated growth of the ER+ cell line ZR-75-1, but the activation peptide-induced stimulation occurred with approximately one day's delay (18).

Cytokines are a group of small glycoproteins whose main function is to act as intercellular signals. They are secreted by and act on lymphocytes, monocyte/macrophages and a variety of other cells, including cells of nonhematopoietic origin. Besides the regulation of immune responses and inflammation, cytokines are heavily involved in numerous physiological and pathophysiological processes. Despite numerous observation, the role of cytokines in cancer development is far from clear. The fact that individual tumors express a wide range of cytokines makes the situation even more complicated. When the expression of 13 different cytokines was investigated in normal and neoplastic human breast tissue, only a difference in expression of IL-8

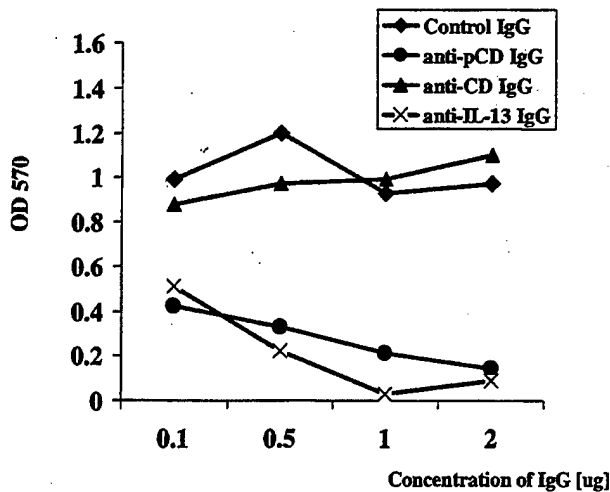


Figure 6. Inhibition of the rIL-13-induced growth of human breast cancer cell line ZR-75-1. The cells were simultaneously incubated with 10 ng of rIL-10 and various concentrations of either anti-procathepsin D IgG, anti-procathepsin D IgG, anti-IL-13 IgG or control MOPC-21 IgG. The results represent the mean of three independent experiments.

transcripts was found (30). The role of cytokines in cancer regulation is still controversial, mostly due to the complexity and pleiotropicity of their biological effects. In breast cancers, the production of IL-3, IL-4 and TGF- β 1 significantly exceeds that detected in benign breast lesions (31).

IL-4 is a highly pleiotropic cytokine executing its effect by interacting with high-affinity IL-4 receptors present on a wide variety of hemopoietic and nonhemopoietic cell types. IL-10 has an extremely wide biological effect reaching from thymocytes to macrophages, B cells and mast cells. IL-13 has a moderate sequence homology (24 to 30%) to IL-4 and shares with it a number of biological activities in the human. Moreover, IL-4 and IL-13 receptors share a common signaltransducing subunit IL-13R/IL-4R α (32) and activate many common signaling pathways.

The presence of various cytokine receptors on human cancer cells is well established. Human breast tumors express IL-4R β and IL-13R α ' (33), all glioma cells express IL-13R α (IL-4 independent subunit of the receptor) (34) and ovarian carcinoma cells express high-affinity IL-4R (35). In addition, levels of some cytokines such as IL-4 and IL-10 have been found to be significantly elevated in patients suffering from various cancers such as prostate (36) or colorectal cancer (37), receptors for IL-4 and IL-13 have been found on head and neck cancer (38) and colon cancer cells (39) or hematological malignancies (40).

Interleukins 4 and 13 strongly inhibited estrogen-induced proliferation of breast cancer cells while they stimulated the GCDPF-15 expression in these cells (41, 42). In addition, IL-4

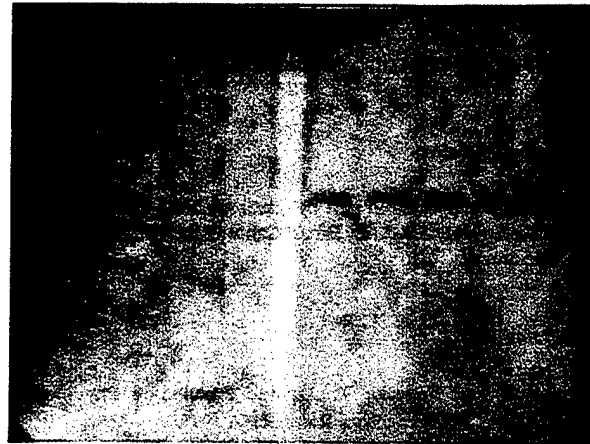


Figure 7. Analysis of the presence of IL-14-induced procathepsin D secretion in tissue culture supernatants by chemiluminescence. Lines (left to right): ZR-75-1 cells with estradiol; MX-2 cells; MDA-MB-231 cells; BT-20 cells; standards; MCF-7 cells; MDA-MB-483 cells; ZR-75-1 cells; procathepsin D diluted 1:10 and procathepsin D.

was shown to induce apoptosis in human breast cancer cells (43). However, the experimental design of these experiments showing interleukin-mediated inhibition used cancer cells cultured in fetal calf serum, thus the real effects might be masked by interaction between lymphokines and serum-derived growth factors. In addition, some of these studies are in fact rather contradictory. Serve *et al.* (42) described inhibition of proliferation and clonal growth of breast cancer cells by cytokines, but in fact IL-4 inhibited the proliferation of only 3 out of 9 cell lines, while IL-13 showed a minor effect (16% of inhibition) in two out of three tested lines. Some of the inhibitory effects are based on changes in down-regulation of E-cadherin and CEA molecules (39).

These three anti-inflammatory cytokines used in our paper were also found to stimulate proliferation of human acute myelogenous leukemia cells (44), suggesting that the role of these cytokines in cancer development is more complex than originally suspected. In addition, some data suggest that endogenous production of cytokines by some cancer cells contributes to their growth and therapeutic resistance (45). Blais *et al.* found no effect on proliferation of breast cancer cells by IL-4 or IL-13 (41).

From our results we can hypothesize that, with respect to procathepsin D secretion in breast cancer, some interleukins act in a similar fashion to estrogens. Due to the fact that procathepsin D functions as an autocrine mitogen, its secretion (regardless of the mechanism) is followed by higher proliferation of these cells. Our study suggests that for the studied cell lines both mechanisms of procathepsin D secretion brought the same effect *i.e.* more aggressive growth of cancer cells. Our experiments support the hypothesis that procathepsin D may play a crucial role in the final stages of

cancerogenesis of E+ cancer cells. These data add credence to the hypothesis that breast cancer development is regulated in part by local autocrine and paracrine mechanisms.

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Detection of procathepsin D in rat milk

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Abstract

The presence of procathepsin D, a zymogen of the soluble lysosomal aspartic proteinase cathepsin D, was detected in rat milk using Western blot analysis and assay of proteolytic activity in acidic buffers. No other forms of cathepsin D were found. Two different polyclonal anti-procathepsin D antibodies were used for immunochemical detection of procathepsin D. Both antibodies we found to recognize rat procathepsin D. Proteolytic activity in acidic buffers was detected using a fluorogenic substrate specific for cathepsin D and was abolished by pepstatin A, a specific inhibitor of aspartic proteinases. This study represents third demonstration of presence of procathepsin D in mammal breast milk. Potential sources and physiological functions are discussed.

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1. Introduction

Procathepsin D, an enzymatically inactive zymogen of the soluble lysosomal aspartic proteinase cathepsin D (E.C. 3.4.23.5), has been demonstrated in human (Vetvicka et al., 1993) and bovine (Larsen et al., 1993) milk. Besides procathepsin D, other cathepsin D forms (pseudo-, single-chained and two chained) have been identified in bovine milk (Larsen et al., 1993) with procathepsin D being the predominant form. On the other hand, no mature cathepsin D was detected in human milk (Vetvicka et al., 1993). These observations lead the authors to suggest that procathep-

sin D is secreted to milk rather than escaped from damaged mammary cells.

The exact origin of procathepsin D in human and bovine milk is not known. It has been shown, however, that procathepsin D can be secreted by mammary epithelial cells (Capony et al., 1989) and that activated lymphocytes can secrete lysosomal enzymes (Hasilik, 1992). However, simple escape from mammary cells cannot be excluded. The mammary gland undergoes extensive changes under influences of several hormones at the onset of lactation. It has been shown that cathepsin D activity in the lactating mammary tissue is 3-fold higher than in controls (Hernandez-Montes et al., 1999). The level of procathepsin D in breast milk could be also affected by the presence of pathological processes.

Besides human and bovine procathepsin D, the complete amino acid sequence is known for other mammals, such as rat (Fujita et al., 1991) and

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mouse (Diedrich et al., 1990). In addition, partial porcine (Faust et al., 1985) and ovine (Tyynela et al., 2000) procathepsin D sequence was also described. So far, however, nothing is known about procathepsin D concentrations and cathepsin D activity in milk of other mammals. In the present study, we report the detection of procathepsin D in rat milk using two different anti-procathepsin D antibodies as well as proteolytic activity at low pH inhibited by pepstatin A, a potent inhibitor of aspartic proteinases.

2. Materials and methods

2.1. Chemicals

Oxytocin, RPMI 1640 medium, HEPES, MTT, antibiotics and pepstatin A were obtained from Sigma (St. Louis, MO), fetal calf serum (FBS) was from Hyclone Laboratories (Logan, UT), and cathepsin D substrate was obtained from Bachem (Torrance, CA).

2.2. Antibodies

Rabbit anti-rat cathepsin D polyclonal antibody was obtained from Wako PureChemical Industries (Richmond, VA), rabbit anti-human procathepsin D polyclonal antibody raised against a synthetic 44-residue propeptide of human procathepsin D was custom-prepared by Genosys Biotechnologies (Woodlands, TX).

2.3. Milk collection

The banked rat milk was collected by manual expression from anesthetized mothers on day 8–12 of lactation. The pups were separated from their mothers approximately 4–6 h before milking. After subcutaneous injection of a mixture of ketamine, xylazine, and acepromazine, the mother rats were given 2 units of oxytocin intraperitoneally (Dvorak et al., 2000). Twenty minutes later, milk was expressed gently into polypropylene tubes and stored at -70°C for no more than 6 weeks before use. Each rat was used only once for the harvesting of rat milk.

2.4. Processing of rat milk

Before experiments rat milk was thawed and centrifuged for 30 min at 10 000 g. Medium phase

was collected and used for Western blotting and cathepsin D activity analysis.

2.5. Cell line

Human breast cancer cell line ZR-75-1 was obtained from American Tissue Culture Collection (ATCC, Manassas, VA). The cell line was grown in RPMI 1640 medium with HEPES buffer supplemented with 10% heat-inactivated FCS, 2 mM L-glutamin, 100 U/ml penicillin and 100 cg/ml streptomycin. To obtain medium for Western blotting analysis, cells were seeded in 2×10^5 cells/ml density. Medium was replaced on the second day with RPMI 1640 medium without FCS and cells were cultivated for 48 h. Conditioned medium was then concentrated 10-fold using Nanosep 10K Omega (Pall Gelman Laboratory, Ann Arbor, MI).

2.6. Western blotting

Rat milk samples and $10\times$ -concentrated conditioned medium from ZR-75-1 cells (serving as a positive control) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Samples were diluted 1:1 in Laemmli sample buffer and heated at 95°C for 4 min (Laemmli, 1970). Electrophoresis of the denatured samples was carried out using 12% Tris-HCl ready precast gel (Biorad) at 200 V for 35 min at room temperature in 25 mM Tris, 190 mM glycine, 0.05% (w/v) SDS, pH 8.3 buffer. Proteins were then electrotransferred to nitrocellulose membrane (0.45 cm) (Biorad) at 100 V for 1 h in 25 mM Tris, 190 mM glycine, 20% (v/v) methanol. After blocking for 1 h in 10 mM Tris, 100 mM NaCl, 0.1% (v/v) Tween 20, pH 7.5 (blocking buffer), the membrane was incubated with custom-prepared rabbit anti-human procathepsin D polyclonal antibody (1:1000 in blocking buffer) for 1 h at room temperature. The membrane was then washed 3 times with blocking buffer and incubated for 1 h with anti-rabbit IgG-alkaline phosphatase conjugate secondary antibody (Sigma) (1:10 000 in blocking buffer). After washing membrane with blocking buffer ($2\times$) and blocking buffer without Tween 20 ($2\times$) pCD-antibody complexes were detected using NBT/BCIP alkaline phosphatase substrate kit (Biorad).

To confirm the specificity of our Western blotting assay, the primary antibody was replaced with rabbit anti-rat cathepsin D polyclonal antibody and

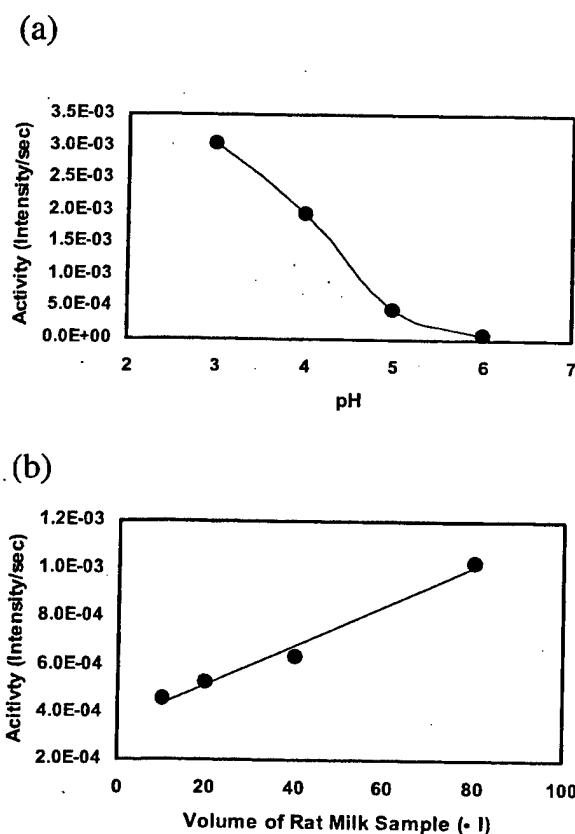


Fig. 1. Assay of rat milk samples using a fluorogenic substrate specific for cathepsin D. (a) Proteolytic activity increases in increasingly acidic buffers. (b) Proteolytic activity increases linearly with the volume of rat milk sample added to the assay at pH 3.

Western blotting assay was performed again as described above.

2.7. Activity of cathepsin D

Activity of cathepsin D was assayed using a fluorogenic substrate specific for cathepsin D (Bachem) according to the method of Yasuda et al. (1999). Briefly, assays were performed with either 0.1 M sodium acetate (pH 3.0) or citrate-phosphate buffers (for pH dependence studies) equilibrated at 37 °C, combined with 5–10 μ l of rat milk sample and 20 μ l of 1 mg/ml substrate dissolved in DMSO. Cleavage of substrate (Yasuda et al., 1999) was followed by excitation at 328 nm and emission at 393 nm using an Aminco AB2 spectrofluorometer. Titration of number of active sites was performed with pepstatin A according to Tomasselli et al. (1990).

3. Results

Assay of rat milk samples using a fluorogenic substrate specific for cathepsin D (Yasuda et al., 1999) revealed an increase of proteolytic activity in increasingly acidic buffers (Fig. 1a). The observed activity was found to increase linearly with the volume of rat milk sample added to the assay at pH 3 (Fig. 1b). To ascertain whether the observed time-dependent change in fluorescence intensity was due to components present in the milk sample other than cathepsin D, inhibition of the activity was attempted with pepstatin A, a potent inhibitor of aspartic proteases (Umezawa et al., 1970). Pepstatin A in the assay in the nM range was found to completely inhibit the activity (data not shown). Although this low concentration of pepstatin A might not cross-inhibit proteases of other mechanistic classes, we sought to determine the minimal amount of pepstatin to inhibit 50% of activity. Pepstatin A in the sub-nanomolar range was found to provide partial inhibition of the activity (Fig. 2). Additionally, the data in that range provided for titration of the number of active sites present in the assay (Tomasselli et al., 1990) by extrapolation of the activity data to zero activity. The concentration of cathepsin D present in the rat milk sample was determined to be 2.9 ± 0.5 μ g/ml. Similar levels were obtained from ELISA assay using the same antibodies as in Western blotting (data not shown).

Immunochemical techniques were used to identify the presence of procathepsin D in rat milk samples. Two antibodies were used for detection of procathepsin to confirm the specificity of this analysis. First, a custom-prepared rabbit anti-

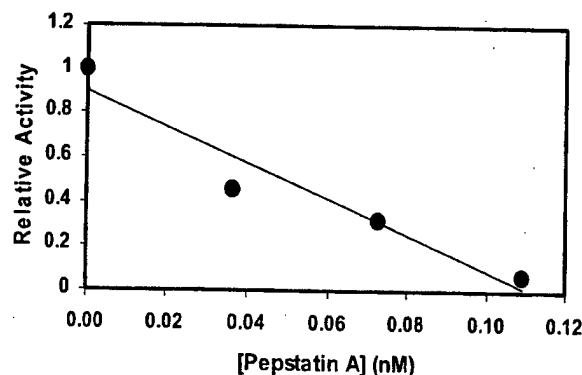


Fig. 2. Inhibition of cathepsin D activity in milk samples by pepstatin A.

human procathepsin D polyclonal antibody was raised against a synthetic 44-residue propeptide of human procathepsin D. Given a high degree of homology between human and rat cathepsin D propeptides (Table 1), this antibody was expected to cross-react between species. This antibody recognized human procathepsin D in ZR-75-1 conditioned media and identified the presence of procathepsin D in samples of rat milk in Western blotting (Fig. 3). Second, a rabbit anti-rat cathepsin D polyclonal antibody was employed and likewise identified procathepsin D in rat milk but only weakly detected human procathepsin D in ZR-75-1 conditioned media (data not shown). Notably, no mature cathepsin D was detected in the samples using these antibodies. As summarized in Table 1, compared to human sequence, there is 70.45% identity with rat, 68.18% with mouse, 70.45% with bovine and 69.23% with ovine procathepsin D, respectively.

Taken together, we showed specific cathepsin D activity in acidic buffers containing samples of rat milk. In addition, rat milk was found to be positive in immunochemical analysis for procathepsin D using two different assays and employing two different anti-procathepsin D polyclonal antibodies. We may conclude that rat milk contains procathepsin D.

4. Discussion

In this study we report that rat milk contains procathepsin D, an inactive zymogen of the aspartic proteinase cathepsin D. No other forms of cathepsin D were detected using immunochemical detection. In the last decade, an increased amount of data has documented an important role of procathepsin D as a prognostic marker (Rochefort and Liaudet-Coopman, 1999) as well as the direct involvement of procathepsin D in cancer growth (Fusek and Vetvicka, 1994; Vetvicka et al., 2000;

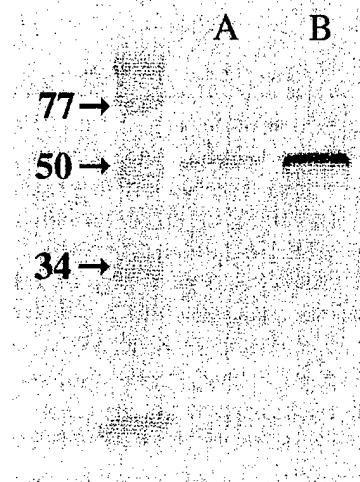


Fig. 3. Western blot of rat milk (A) and tissue culture media (B) from human ZR-75-1 cell line. Procathepsin D was visualized using anti-human procathepsin D polyclonal antibody (Section 2).

Bazzett et al., 1999). In our laboratory we found that procathepsin D has a growth factor activity for human breast and prostate cancer cells (Fusek and Vetvicka, 1994; Vetvicka et al., 2000). We found that growth factor function of procathepsin D is mediated through an unknown specific receptor expressed on breast and prostate cancer cells (Vetvicka et al., 1997, 2000). The parts of the procathepsin D molecule responsible for its mitogenic activity were localized near amino acids 27–44 of the activation peptide sequence (Vetvicka et al., 1997).

In this study, we used rabbit anti-human procathepsin D polyclonal antibody raised against the propeptide sequence of human procathepsin D for immunochemical detection of procathepsin D in rat milk samples. Table 1 shows a comparison of procathepsin D activation peptide sequences in different mammals including human and rat.

Table 1
Propeptide sequences of procathepsin D of different mammals

| | |
|---------|--|
| Human | LVRIPLHKFTSIRRTMSEVGGSVEDLIAKGPVSKYSQAVPAVTE |
| Rat | LIRIPLRKFTSIRRTMTEVGGSVEDLILKGPIPKYSMQSSPRTK |
| Mouse | IIRIPLRKFTSIRRTMTEVGGSVEDLILKGPIPKYSMQSSPKTT |
| Bovine | VIRIPLHKFTSIRRTMSEAMGPVEHLIAKGPISKYATGEPAVRQ |
| Ovine | _____LHKFTSNRRMTSEAMGPVEHLIAKGPISKYATREPVRQ |
| Porcine | LLIPLK_____IM_____P_____LILKILK_____ |

Dashes in the ovine and porcine sequences indicate that amino acid was not determined. Data from Larsen et al. (1993), Fujita et al. (1991), Diedrich et al. (1990), Faust et al. (1985), Tynnela et al. (2000) were used.

Although there are some differences in amino acid sequences of human and rat procathepsin D pro-peptide we were able to detect a positive signal of procathepsin D in rat milk. The confirmation of the presence of procathepsin D in rat milk was done in a second experiment with rabbit anti-rat cathepsin D polyclonal antibody. The amino acid sequence of procathepsin D is well conserved among species and exhibits a high degree of similarity with other aspartic proteinases, e.g., prochymosin and pepsinogen (Hasilik and Neufeld, 1980).

We also showed proteolytic activity of rat milk samples in increasingly acidic buffers consistent with an acidic proteolytic activity optimum of rat cathepsin D for the fluorogenic substrate we used (Yasuda et al., 1999). The presence of cathepsin D activity was further substantiated by inhibition with pepstatin A, a potent inhibitor of aspartic proteinases (Umezawa et al., 1970). Whereas only procathepsin D was evident from western blots of rat milk samples (Fig. 3), the observed cathepsin D activity may be from pseudocathepsin D, an *in vitro* autocatalytically activated cathepsin D (Nishimura et al., 1987; Conner and Richo, 1992). Furthermore, a procathepsin D that was incapable of autoactivation by mutagenesis of the autoactivation site was however observed to be active toward synthetic substrates (Wittlin et al., 1999). Therefore, the activity observed in the rat milk samples is likely from either procathepsin D or from pseudocathepsin D that was formed during the assay.

This study represents the third example of presence of procathepsin D in milk of different mammals. Our group previously found procathepsin D in human milk (Vetvicka et al., 1993) and Larsen et al. reported that procathepsin D represents predominant form of this aspartic proteinase in bovine milk (Larsen et al., 1993). It is certainly of interest that procathepsin D has been demonstrated in milk of all species tested. Due to the known differences in mammary gland architecture between these species, it is possible to suggest an active release of procathepsin D into the milk. At the same time, a fraction of procathepsin D is also released into the blood stream. As activation peptide of procathepsin D has been found to be immunogenic (Vetvicka et al., 1993), low level of immunization and memory cell formation can occur. One can further speculate that in case a breast-feeding individual later develops a procathepsin D-secret-

ing type of breast tumor, her immune system is better prepared to fight the cancer. The presence of procathepsin D in milk just might be much more important than we originally thought. In addition, Saftig et al., while describing cathepsin D-deficient mice, also speculated that cathepsin or even procathepsin D in milk might play a role in early survival of cathepsin $-/-$ mice. However, no proof supporting this possibility has been found (Saftig et al., 1995).

It is well established that maturation of lysosomal enzymes occurs only after segregation from the secretory pathway, therefore the presence of procathepsin D in milk is rather due to the active secretion than to the release from ruptured cells. Procathepsin D can also be released due to the mechanical processes during reproductive cycle. No physiological function of procathepsin D in mammal milk is known, but it has been shown that procathepsin D can play a role in milk protein degradation (Hurley et al., 2000). In addition, human procathepsin D is able to activate neutrophils and lymphocytes by regulation of surface receptors (Vetvicka and Fusek, 1994). These effects are mediated through activation peptide of procathepsin D (Vetvicka et al., 1995). A possible function of this activation in the immune defense of the breast-fed newborn has been suggested (Vetvicka and Fusek, 1994). Interestingly, El Messaoudi et al. recently reported that human immunodeficiency virus type 1 growth in lymphocytes was increased when incubated in breast milk but that the enhancing effect of milk was abolished by anti-cathepsin D antibody or pepstatin A. Authors suggested that cathepsin D might react with viral gp120 and modify its affinities for co-receptors (El Messaoudi et al., 2000).

In summary, to our knowledge all breast milk from different mammals studied so far contain procathepsin D. Physiological function and source of this protein in milk remains to be established.

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